THE EFFECT OF THE ADMINISTRATION OF ACID AND ALKALINE SALTS UPON THE ASCORBIC ACID CONTENT OF GUINEA PIG TISSUES

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In a previous study of the urinary excretion of ascorbic acid (vitamin C) by normal human subjects, it was observed that the administration of acid and alkaline salts was followed by striking variations in the amount of ascorbic acid found in the urine (Hawley, Frazer, Button and Stephens, '36). In subjects who had been previously saturated with vitamin C, the administration of sodium bicarbonate was followed by the excretion of approximately 50% of daily test doses of 100 mg. of ascorbic acid. The substitution of ammonium chloride for sodium bicarbonate resulted in the excretion of a highly acid urine and increase in the daily excretion to approximately 100% of the daily dose of ascorbic acid.

In a preliminary experiment seventy guinea pigs, part of which had been maintained for several weeks on a diet partially deficient in vitamin C during the course of another study, were divided into groups and were given the crystalline ascorbic acid, the ascorbic acid together with $NaHCO_3$ or NH_4Cl or the salts alone, as indicated in table 1.

In all experiments the animals were sacrificed exactly 2 hours after the administration of the ascorbic acid which was given exactly 1 hour after the $NaHCO_3$ or NH_4Cl. The animals were killed and the livers analyzed for ascorbic acid according to the method outlined below. The average amount

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of ascorbic acid found in the liver in each group is shown in table 1.

These observations suggested that retention of ascorbic acid in the liver was increased by the administration of the alkaline salt. The following experiment was designed to further investigate the possible relation of the administration of acid and alkaline salts to the amount of ascorbic acid retained in the liver and adrenals of the guinea pig.

TABLE 1

Ascorbic acid in milligrams per 100 gm. liver

'Positive controls' of original experiment—normal animals

Control group	11.25
Ammonium chloride	11.08
Ammonium chloride + 10 mg. ascorbic acid	11.20
Sodium bicarbonate	11.15
Sodium bicarbonate + 10 mg. ascorbic acid	17.55

Animals on partially deficient diets

$NaHCO_3$ GROUP		$NHCl_4$ GROUPS	
Negative controls	6.63	.....	
5 mg. ascorbic acid [1]	7.28	5 mg. ascorbic acid	7.68
$NaHCO_3$ + 5 mg. ascorbic acid	9.18	NH_4Cl + 5 mg. ascorbic acid	8.42
Negative controls	6.60	Negative controls	6.08
10 mg. ascorbic acid	9.57	10 mg. ascorbic acid	8.02
$NaHCO_3$ + 10 mg. ascorbic acid	11.80	NH_4Cl + 10 mg. ascorbic acid	7.53

[1] In this series the crystalline ascorbic acid was administered rather than the salt.

METHOD

Guinea pigs weighing from 200 to 300 gm. were placed on the Sherman vitamin C deficient diet [1] for a period of 17 days. This was considered a sufficient period of time to deplete the vitamin C stores and induce mild scurvy without resulting in marked pathological changes. At the end of the depletion

[1] Wheat bran 29.5 parts; ground rolled oats 29.5 parts; skim milk powder (heated in shallow trays for 5 hours at 110°C.) 30 parts; sodium chloride 1.0 part; butter fat 8.0 parts; cod liver oil 2.0 parts.

period the animals were divided into six groups as indicated below:

Group A, killed at the end of the 17-day depletion period.

Group B, fed 10 mg. of ascorbic acid daily. Killed in sub-groups after 2, 4, 6, 15 and 23 days.

Group C, fed 10 mg. of ascorbic acid and 32.5 mg. of ammonium chloride daily. Killed in sub-groups after 2, 4, 6 and 15 days.

Group D, fed 10 mg. of ascorbic acid and 100 mg. of sodium bicarbonate daily. Killed in sub-groups after 2-, 4-, 6-, 8-, and 15-day periods.

Group E, adequate intake of C for 40 days (17 + 23 days).

The sixth group of guinea pigs, group F, was fed without previous depletion a diet adequate in vitamin C, with an added daily ration of 5 cc. of orange juice for a period of 45 days, at the end of which time they too were sacrificed.

At the end of each experimental period the animals were chloroformed, autopsied and examined according to the method of Sherman, LaMer and Campbell ('22). The degree of scurvy in each animal was expressed numerically as the sum of the plus signs recorded in the scoring. The tissues were quickly removed, weighed, ground with fine sand and extracted by vigorous shaking for 5 minutes with a 20% acid mixture (16% trichloracetic acid, 4% metaphosphoric acid). Sufficient water was then added to reduce the acidity to 5% and the acid tissue mixture was shaken for 10 additional minutes, after which it was filtered. The filtrate was titrated against a standardized solution of 2, 6-dichlorobenzenoneindophenol.[2] The results were expressed as milligram per cent ascorbic acid. The hydrogen ion concentration was determined by the colorimetric method in urine aspirated from the bladder at the time of autopsy.

RESULTS

Marked individual variations in the ascorbic acid content of guinea pig tissues were observed. It is sometimes assumed that results obtained by the chemical method in controlled groups of animals are more or less uniform. Table 2 is inserted to illustrate the wide variations which are found

[2] Practical substitute for 2, 6-dichlorphenolindophenol, Eastman Kodak Co.

TABLE 2

Ascorbic acid content of guinea pig tissues reported in the literature

INVESTIGATORS	DIET	NUMBER OF ANIMALS	MILLIGRAMS ASCORBIC ACID PER 100 GM.	
			Adrenal	Liver
Diets adequate in vitamin C				
Bessey and King ('33)	C free + excess spinach for 10 days	?	75	10
Yavorsky, Almaden and King ('34)	C free + excess spinach for 10 days	8	70	10
Birch and Dann ('33)	Normal diet	?		30
Svirbely ('33)	C free + spinach for 21 days	4	124 (114–135)	23 (11–32)
Fox and Levy ('36)	Cabbage and bran	10	150 (110–206)	25
Fox and Levy ('36)	Same + 100 mg. ascorbic acid for 8 days	2	201 (196–206)	40 (38–42)
Jacobsen ('35)	C free diet + 20 mg. ascorbic acid for 9 days	2	38	7.7
Jacobsen ('35)	Same for 3 months	2	18	6.2
Jacobsen ('35)	Same + cabbage ad lib for 3 months	2	78	25
Hawley, Daggs and Stephens	Adequate diet + 5 cc. orange juice for 45 days	5	39 (22–77)	25 (11–28)
Diets inadequate in vitamin C				
Bessey and King ('33)	C free for 15 days	?	10	4.3
Yavorsky, Almaden and King ('34)	C free for 15 days	6	8	3
Yavorsky, Almaden and King ('34)	C free for 28 days	6	3	1
Birch and Dann ('33)	'Scorbutic'	?		9
Svirbely ('33)	C free for 21 days	4	7 (6–10)	2.0
Hawley, Daggs and Stephens	C free for 17 days	10	12.6 (9–16)	2.7 (2–4)

in the literature. Table 3 shows the variations encountered in the individual animals within a single group, the 'F' group. These animals had received a diet of alfalfa hay, whole oats and cabbage. To this was added a daily ration of 5 cc. of orange juice in an attempt to completely saturate the tissues with vitamin C. The saturation point for individual animals evidently varied within wide limits. The ascorbic acid content of the tissues of this group of animals showed greater variations than any of the other groups. This, we feel, emphasizes the value of preliminary standardization by depletion in a study such as this before the experimental diet is fed.

The results summarized in tables 4 and 5 appear to confirm the observations of the preliminary experiment. They indicate that the administration of sodium bicarbonate with

TABLE 3

Variations in ascorbic acid content of organs from animals within the same test group (F)

Animal no.	1	2	3	4	5	AVERAGE
						mg.%
Liver	27.9	16.0	14.2	11.3	10.0	16.02
Adrenal	21.5	76.5	41.0	32.1	25.9	39.40

ascorbic acid increases the retention of the vitamin. They further suggest that when the vitamin is ingested in its natural form as cabbage, alfalfa hay and orange juice (F group) that there is even better vitamin retention by the tissues. Jacobsen ('35) likewise found in his series that the tissue content of those animals receiving the basal diet plus cabbage ad lib (equivalent to approximately 20 mg. ascorbic acid daily) was definitely higher in ascorbic acid than was the tissue of the animals receiving the equivalent amount of ascorbic acid in its crystalline form.

After the sixth day, the adrenals and, to a lesser extent, the livers of those animals which had received sodium bicarbonate contained significantly larger amounts of ascorbic acid than those animals which had received ammonium chloride or those which were given ascorbic acid alone. Furthermore, the

TABLE 4

Summary of present experimental data

GROUP	CONDITION	TEST DAYS	NUMBER OF ANIMALS	MILLIGRAMS PER CENT ASCORBIC ACID		COMPOSITE URINARY pH	AVERAGE SCURVY SCORE	WEIGHT CHANGE IN GRAMS
				In liver	In adrenals			
A	C free diet	17	10	2.65 (1.58–4.03)	12.46 (9.30–15.50)		9	— 6
B	Ascorbic acid added	2	9	6.92 (5.13–8.43)	14.13 (11.45–17.20)		12	— 33
		4	9	6.94 (4.43–10.90)	17.83 (9.10–25.00)	6.9	10	— 25
		6	9	7.84 (5:35–10.15)	21.36 (14.95–33.20)		7	— 10
		15	7	8.01 (7.00–9.40)	16.88 (9.30–24.10)	6.8	1—	+ 21
C	Ascorbic acid + NH_4Cl	2	8	7.79 (7.10–9.75)	13.92 (12.05–17.10)	6.0	13	— 24
		4	9	5.85 (5.00–7.50)	16.92 (12.00–21.40)	6.1	9	— 27
		6	8	6.87 (4.60–9.75)	21.34 (11.55–36.20)	6.4	11	— 21
		15	8	7.36 (4.80–10.00)	15.04 (8.60–33.40)	5.6	1—	+ 68
D	Ascorbic acid + $NaHCO_3$	2	3	7.10 (5.85–8.80)	15.27 (13.90–17.70)	7.4	18	— 36
		4	6	4.81 (3.25–6.35)	16.68 (14.00–20.90)	8.0	8	— 4
		6	5	8.64 (5.05–13.25)	34.12 (19.90–46.80)		3	— 14
		8	5	10.42 (6.92–15.86)	25.32 (18.40–33.80)	7.8	1	+ 21
		15	7	10.03 (7.00–12.60)	32.07 (26.20–40.60)	8.0	1—	+ 48
E	Adequate diet	40	5	10.15 (5.50–17.70)	20.71 (10.50–35.00)	7.6	0	+ 100
F	Adequate C + orange juice	45	5	16.02 (10.70–27.90)	39.40 (21.50–76.50)	8.0	0	+ 150

scurvy scores suggest that the additional ascorbic acid retained by those animals which received the alkaline salt was utilized to prevent or correct manifestations of scurvy. The changes in the urinary hydrogen ion concentration were comparable to those observed in human subjects (Hawley, Frazer, Button and Stephens, '36).

TABLE 5

Summary of average values for various organs in the A, E and F groups

GROUP	LIVER	ADRENAL	KIDNEY	HEART	SPLEEN	BRAIN	EYES	OVARIES	TESTES	BLADDER	SCURVY SCORE	NUMBER ANIMALS	URINE pH
A[1]	2.65	12.46	1.92	2.86	5.97	3.99	2.59		3.91	10.62	9	10	?
E[2]	10.15	20.71	5.04	3.54	7.21	11.94	3.94	17.00	16.30	10.25	0	5	7.6
F[3]	16.02	39.40	7.90	4.70	18.00	7.48					0	5	8.0

[1] C free diet.

[2] Adequate diet.

[3] Adequate diet plus orange juice.

SUMMARY

In a previous study the authors observed a definite relationship, in humans, between the amount of ascorbic acid found in the urine and the hydrogen ion concentration of the latter. Observations in guinea pigs show that the administration of sodium bicarbonate in amounts sufficient to result in a highly alkaline urine results in an increased concentration of ascorbic acid in the adrenals and liver. It is shown that increased retention of ascorbic acid in the tissues may be at least a partial explanation of the decreased urinary output of ascorbic acid observed in humans in whom the urine had been rendered alkaline by the administration of sodium bicarbonate.

The authors wish to take this opportunity to express their appreciation to Merck & Company who generously supplied the ascorbic acid used in this experiment.

LITERATURE CITED

BESSEY, O. AND C. C. KING 1933 The distribution of vitamin C in plant and animal tissue and its determination. J. Biol. Chem., vol. 103, p. 687.

BIRCH, T. W. AND W. J. DANN 1933 Evaluation and distribution of ascorbic acid and glutathione in animal tissues. Nature, vol. 131, p. 469.

FOX, F. W. AND L. F. LEVY 1936 Experiments confirming the antiscorbutic activity of dehydroascorbic acid and a study of its storage and that of ascorbic acid by the guinea pig at different levels of intake. Biochem. J., vol. 30, p. 211.

HAWLEY, E. E., J. P. FRAZER, L. L. BUTTON AND D. J. STEPHENS 1936 The effect of the administration of sodium bicarbonate and of ammonium chloride on the amount of ascorbic acid found in the urine. J. Nutrition, vol. 12, p. 215.

JACOBSEN, ERIC 1935 On the storage of ascorbic acid in the organs of guinea pigs given crystalline ascorbic acid with a vitamin C free diet. Skand. Arch. Physiol., vol. 72, p. 259.

SHERMAN, H. C., V. K. LAMER AND H. L. CAMPBELL 1922 The quantitative determination of the antiscorbutic vitamin. J. Am. Chem. Soc., vol. 44, p. 165.

SVIRBELY, J. L. 1933 The ascorbic acid content of the adrenals and livers of different animals. Biochem. J., vol. 27, p. 960.

———— 1935 The effect of desiccated thyroid and α dinitrophenol, and cortical hormone extract on vitamin C content of some organs of the guinea pig fed graded doses of ascorbic acid. J. Biol. Chem., vol. 111, p. 147.

YAVORSKY, M., P. ALMADEN AND C. G. KING 1934 Vitamin C content of human tissues. J. Biol. Chem., vol. 106, p. 525.

COMPARATIVE EXPERIMENTS WITH CANNED, HOME COOKED, AND RAW FOOD DIETS[1]

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Much has been said and written about the relative merits of raw and cooked foods. Probably the difficulty of securing experimental evidence on such a question is the reason for the limited concrete data. Three comprehensive series of animal experiments are herewith presented. The first was conducted over a number of successive generations. To eliminate a possible hereditary effect a second plan limited to one generation was followed. That any variable due to variation in lacteal performance might be eliminated, a third was limited to the period of rapid growth after weaning.

Vitamins bring cooking into question

With the discovery of the vitamins, there came a questioning of the cooking of foods. The canning of foods generally requires, for purposes of sterilization, more thorough cooking than is otherwise practiced. Studies on the effect of the canning process on the various vitamins in our most common vegetables and fruits have shown that cooking need not be deleterious to vitamins. In fact, insofar as canning serves to promote a greater consumption of vegetables and fruits, it tends to enhance our vitamin intake. Tressler, Mack and King ('36) recently conclude that in cooking "the total amount

[1] Presented before the Division of Biological Chemistry, American Chemical Society, Chapel Hill, North Carolina, April, 1937.

of ascorbic acid decreases but little," calling attention at the same time to the fact that "a considerable proportion of the ascorbic acid passes into the water during the boiling of vegetables." In the experiments here recorded the cooking water was held to a minimum and fed with the food cooked in it.

Initial experiment with all canned food diet

A study of the vitamins cannot, however, give a comprehensive answer to the question of the effect of cooking foods, since the necessary dietary constituents comprise a great many other factors—some undoubtedly as yet not recognized. Physical factors such as texture, consistency, colloidal conditions, etc., and possibly the activity of enzymes are also involved. It was therefore decided to feed two species of animals—guinea pigs and rats—a diet made up wholly of canned foods through a number of generations. While it may be argued that man, through his experience over many centuries, has undergone physiological changes to adapt himself to cooked foods, such a statement would not be pertinent to these two species of animals. If canning adversely affects the nutritive value of foods, it would be expected to show up after a few generations. No such evidence developed. As a matter of fact, the guinea pigs through eight generations, and the rats through ten generations, thrived better than usual. Growth rates and weights were above those recorded as average (Kohman, Eddy and Gurin, '31).

Plan for feeding canned, home cooked, and raw foods comparatively

The outcome of this experiment suggested the desirability of conducting more comprehensive experiments in which canned foods, ordinary (home) cooked foods, and raw foods were to be fed on a comparable basis. Rats have been used in such experiments. They were planned to conform as nearly as possible to the consumption of foods under usual conditions. The junior author (M.W.) in charge of the animals

was instructed to purchase all foods off the New York market just as they would be purchased for household use, and cook those for the home cooked diet according to directions obtained from the Foods and Cookery Department of Teachers College. The same group of foods was always purchased for each of the three diets. It should be borne in mind that the raw and home cooked diets represented the same raw products since purchases off the New York market were simply divided to constitute the two diets. The canned food diet represents different lots of the same food and there was no record of where or when they were canned except that they represented canned foods on the market at the time. Each group of foods used comprised different types of food to provide what is considered a balanced meal. To simplify matters, a given group of foods was usually employed for a period of a few days to a week. Milk was not used because of its ability to cover up dietary deficiencies. Food mixtures and proprietary products, the components of which were uncertain, were avoided. Typical groups of combinations of foods used are:

Roast beef, white potatoes, green beans, tomatoes, peaches
Tongue, sweet potatoes, peas, pumpkin, blueberries
Chicken, noodles, okra, turnip greens, strawberries

In about fifty similar combinations, practically all the common vegetables, fruits, meat and fish products were used. All the experiments conducted on this general subject in different ways have covered a period of over 4 years and therefore took into account any seasonal variation.

These combinations were fed to the experimental animals by putting each food in a separate dish and allowing each animal a free choice. Grinding or macerating the food for mixing into an homogeneous mixture did not seem warrantable because in the case of raw foods rapid enzymic changes would be induced. Moreover, maceration is part of the digestive process. There is considerable evidence that animals have the ability to choose diets to accomplish optimum results if suitable foods are available. Davis ('28) obtained

excellent results by allowing infants a free choice of their foods. Franke and Potter ('36) found that rats always chose a normal wheat in preference to one grown on seleniferous soil and containing selenium to the extent of 30 parts per million. In case of mixtures of the two the mixtures containing the least selenium-bearing wheat were chosen.

The evidence of five generations

Our first plan of experimentation was to start three pairs of rats on each diet, using litters of weaning age and distributing them between the diets as equally as possible both as to

TABLE 1

Reproductive record of rats on raw, home cooked and canned food

	FOOD		
	Raw	Home cooked	Canned
Total number of litters[1]	67	51	60
Litters dead before weaning, per cent	12	6	5
Number of young at birth	476	405	482
Young dead before weaning, per cent	22.2	12.4	12.00
Average number per litter at birth	7.10	7.94	8.00
Average weight of young at birth, grams	4.68	4.96	5.28
Average weight of young when weaned, grams	20.5	23.0	27.3
Average weight when 60 days old	73	71	89
Average weight when 90 days old	125	125	149

[1] The home cooked group were started somewhat later than the other two and hence not with litter mates of the other two but of the same stock. Hence total numbers cannot be compared with the other two groups.

weight and as to sex. It should be understood, however, that the group on home cooked foods was started somewhat later than the other two as an after thought. Total numbers are therefore not to be compared with similar numbers of the other two groups. This group, while not started with litter mates of the other two, did originate from the same stock. Through five generations close to 500 animals were produced on each diet. The records of this experiment are given in table 1 which is abbreviated from the original report (Kohman, Eddy and associates, '34).

It was particularly interesting that a determination of the percentage of bone ash of the alcohol-ether extracted dried tibia of a considerable number of animals disclosed a distinctly higher degree of calcification resulting from a canned food diet. This point was therefore made the subject of the experiments recorded below.

Single generation experiments to eliminate influence of heredity

One weakness in the above line of experimentation is that all the animals on each diet originated from three pairs. Even though these pairs represented litter mates, as was the case of animals on canned and raw foods, respectively, it might still be argued that heredity might have played a considerable role. To meet this weakness, an entirely different procedure was employed. Litter mates were again used to represent the different diets. Mature animals were bred so as to obtain at least three litters on the same day. The young of each of the three litters were then equally divided between the three mothers, and one mother with its young placed on raw foods, another mother with its young on a portion of these same foods after kitchen cooking and the third mother with its young on a canned food diet. When the young were weaned at 21 days of age, they were kept on their respective diets until 60 days of age for both growth and bone ash records. In a subsequent experiment, the particular mother that had reared her young on a raw food diet, for example, was placed on one of the other two diets and in still another experiment, on the third diet, so as to bring about uniformity of animals on the three diets as far as possible. In experiment 5, table 2, in addition to dividing the young of each litter on the day of birth between the three mothers, at the end of the first week the mothers but not the young were shifted to one of the other two diets and at the end of the second week to the third diet. Fortunately, the maternal characteristics of the animals permitted this without any disturbance. The various experi-

ments conducted with this procedure are summarized in table 2.

The outstanding fact from these data is the distinctly better or higher percentage of bone ash resulting from the canned

TABLE 2

Average bone ash of the alcohol ether extracted dried tibia of 60-day-old animals on canned, home cooked and raw food diets, respectively

EXPERIMENT NO.	DATE OF EXPERIMENT AND DIET	NUMBER OF ANIMALS	AVERAGE WEIGHT OF ANIMALS	BONE ASH OF TIBIA	PROBABILITY OF NON-SIGIFICANCE OF DIFFERENCE IN BONE ASH BETWEEN ANIMALS ON CANNED FOODS AND	
					Home cooked foods	Raw foods
1	July 7, 1934		gm.	%		
	Canned	9	110	42.0	< 0.01	< 0.01
	Home cooked	9	106	36.6		
	Raw	9	101	36.9		
2	July 10, 1934					
	Canned	8[1]	76	37.9	0.10	0.74
	Home cooked	9	81	35.4		
	Raw	9	89	37.5		
3	Oct. 15 1934					
	Canned	6	168	51.2	0.15	< 0.01
	Home cooked	4[1]	199	50.1		
	Raw	5[1]	174	45.4		
4	Oct. 16, 1934					
	Canned	9	112	49.6	< 0.01	< 0.01
	Home cooked	9	98	43.8		
	Raw	9	92	40.9		
5	March 4, 1935					
	Canned	6	140	48.4	< 0.01	< 0.01
	Home cooked	6	154	45.3		
	Raw	6	131	43.1		
6	March 4, 1935					
	Canned	8	153	51.0	< 0.01	< 0.01
	Home cooked	8	130	44.4		
	Raw	8	126	47.0		

[1] The reason for unequal numbers of animals in this experiment is due to deaths before weaning time on these diets.

food diet, comparable to what was manifest in the previous line of experimentation. There is apparent a fluctuation in the weight attained by the animals on the three respective diets which may be no more than the normal variations to be

expected. At any rate, there is no consistent difference in weights attained on the three diets.

The weight up to this age (60 days) is not independent of the nursing period during which only the mothers eat of the respective diets. There is no means of knowing how much variation in lacteal performance of the mother might have affected the results. Whatever the weight attained, there is a striking consistency in the higher percentage of bone ash in the animals on the canned food diet. Another weakness in this plan of experimentation is that although the three mothers were given an equal number of young in the beginning of each experiment, an occasional death occurred before the weaning period. Since during this period the young animals depend almost exclusively on the milk of their mother, this reduction of the number of the litter gave the remaining animals an advantage. This is clearly reflected in the weight of the animals in experiment 3 in table 2. As is apparent from the data, no deaths occurred from the litter on the canned food diet which might have worked to the advantage of the remaining animals since that would leave a smaller number to subsist on the milk of the mother. In spite of this, the average percentage of bone ash is distinctly higher for those animals reared on canned foods. The greater weight attained on each diet in this experiment than in the others no doubt reflects lacteal advantage of small litters.

Experiments covering rapid growth period after weaning

To meet the weakness of this line of experimentation, another series of experiments was conducted on still another plan. Stock animals were taken from the Teachers College, Columbia University colony at a rather early weaning period of 21 days of age. Litter mates were again divided for uniformity both as to sex and weight between the three diets to secure six animals on each diet for each of six experiments covering a period of about a year. These were all fed to an age of 60 days, when the growth and bone ash records were obtained. The data are summarized in table 3.

While there is some fluctuation between the raw and home cooked food animals, again in each experiment there is a consistently higher percentage of bone ash in those animals receiving the canned food diet.

TABLE 3

Average bone ash of the alcohol ether extracted and dried tibia of six 60-day-old animals on canned, home cooked and raw food diets, respectively, in six separate experiments

EXPERIMENT NO.	DATE OF EXPERIMENT AND DIET	AVERAGE WEIGHT OF ANIMALS	BONE ASH OF TIBIA	PROBABILITY OF NON-SIGNIFICANCE OF DIFFERENCE IN BONE ASH BETWEEN ANIMALS ON CANNED FOODS AND	
				Home cooked foods	Raw foods
7	June 20, 1935	*gm.*	%		
	Canned [1]	92	42.7	0.10	< 0.01
	Home cooked [2]	106	40.9		
	Raw	85	38.9		
8	June 22, 1935				
	Canned [3]	99	43.6	< 0.01	0.018
	Home cooked	103	38.8		
	Raw	99	40.5		
9	Nov. 29, 1935				
	Canned	101	47.5	< 0.01	0.26
	Home cooked	121	42.6		
	Raw	108	44.9		
10	Dec. 9, 1935				
	Canned	104	49.7	0.015	< 0.01
	Home cooked	112	43.0		
	Raw	106	43.4		
11	Jan. 20, 1936				
	Canned	128	51.3	< 0.01	< 0.01
	Home cooked	116	44.8		
	Raw	121	46.7		
12	Jan. 27, 1936				
	Canned	128	51.2	< 0.01	0.04
	Home cooked	131	42.5		
	Raw	130	47.5		

[1] Two of six animals discarded because of severe cold and loss in weight.

[2] Two of six animals died; one apparently of polyneuritis, the other of pneumonia.

[3] One of six animals died apparently of pneumonia.

COMMENTS

It may be said that the data presented in these various tables are of an empirical nature and present no evidence in explanation of the results. The data are, however, voluminous enough to be significant. This is apparent from a statistical treatment by Students method of the difference between the per cent of bone ash of animals receiving canned foods and home cooked market foods, respectively, as one comparison and between canned and raw foods as a second comparison. It is obvious there is no significant difference between the results from feeding home cooked and raw foods respectively. The results of statistical treatment are given in the last column of tables 2 and 3. From this it is apparent that in these experiments involving 240 animals with an average of six and two-thirds animals per diet in each experiment—minimum four, maximum nine—the chance that the results are non-significant is less than one in 100 in sixteen out of twenty-four comparisons, and of the remaining eight it is less than five in 100, a reasonable criterion for probability of significance.

Some plausible explanations of these results can be postulated although some of the theories that can be advanced in explanation would be difficult to put to an actual test. It is hoped, however, that in time further explanatory data may be obtained. Certainly the idea that a certain amount of raw foods is absolutely indispensable to health might well be questioned in the face of such evidence. Probably the advocacy of a complete raw food diet has only come from faddists. To consider this question on a rational basis, it should be borne in mind that each dietary essential is a chemical compound or combination of chemical compounds. If cooking does not alter these to prevent their playing their particular role in nutrition, cooking from this standpoint cannot be decried. Cooking inactivates plant enzymes. Their activity in a ruptured raw plant cell is generally not wholesome That conditions should be such in the digestive tract as to make it wholesome is scarcely to be expected.

In these relatively short experiments there is apparent no consistent difference in weight attained as was evident in the earlier experimental procedure in which the dietary regime was maintained over a considerable number of generations. It must be borne in mind that cooked food is a novelty that does not always make an immediate appeal to these animals. Autopsy of a number of animals revealed the fact that the animals receiving raw foods tended to have enlarged caecum and a greater amount of food in the digestive tract and this would be reflected in the weights particularly of young animals. The so-called 'home cooking' is only mild compared to that required of canned foods for sterilization purposes. The raw foods employed, being market produce, must necessarily have been subjected to more or less storage that enhances the fibrous material and is known not to be favorable to vitamin content, which is also affected more or less by the cooking process. Hence little difference between the raw and home cooked diet is not illogical.

The most apparent effect of cooking foods is on their physical structure and texture. While nutritionists have given great emphasis, by their investigations, to the chemical composition of the diet, clinical experience must be turned to for most of the available evidence on the effect of physical structure and texture. It is believed that there is a great deal in the data presented herewith that conforms with this clinical experience.

For those who are familiar with the absorptive properties of certain vegetable fibers toward calcium, there is a plausible explanation for the better utilization of calcium as a result of thorough cooking. In a diet in which the calcium content is below optimum, thorough cooking if it increases calcium availability should effect a higher percentage of bone ash. To illustrate, peas rather rapidly take calcium out of water when suspended in it. Excessively hard water cannot be used in canning peas because this absorbed calcium causes toughening of the skins. The absorption of calcium by the pea skins is a reversible process and the calcium may again be removed

by salt solution. In other words, the pea skins function similarly to a zeolite water softener. The diets used in these experiments, since they did not include milk, probably did not provide an optimum calcium intake. Any factor affecting calcium utilization would thus readily manifest itself. Since cooking tends to break down vegetable tissue, it would tend to lower the absorptive effect of the vegetable fiber for calcium and thus increase calcium availability.

CONCLUSION

Objective evidence from each of three series of experiments indicates there is no inherent virtue in 'rawness.' Furthermore cooking need not incapacitate nutrient elements. It does have beneficial effects on physical structure and texture of foods and inactivates plant enzymes which are detrimental in many instances in handling foods. In so far as canned foods represent the raw product in their prime, they represent a unique means of distributing perishable foods with their inherent nutrient qualities efficiently conserved. Their vitamin content has previously been dealt with and it is here shown that they afford an efficient form of calcium.

LITERATURE CITED

DAVIS, CLARA M. 1928 Self selection of diet by newly weaned infants. Am. J. Dis. Child., vol. 36, p. 651.

FRANKE, KURT W. AND VAN R. POTTER 1936 The ability of rats to discriminate between diets of varying degrees of toxicity. Science, vol. 83, p. 330.

KOHMAN, E. F., W. H. EDDY AND CELIA ZALL GURIN 1931 Vitamins in canned foods. XI. A canned food diet. Ind. Eng. Chem., vol. 23, p. 1064. Also previous papers in this series.

KOHMAN, E. F., W. H. EDDY AND ASSOCIATES 1934 Vitamins in canned foods. XIV. Calcium and vitamin D in foods. Ind. Eng. Chem., vol. 26, p. 758.

TRESSLER, D. K., G. L. MACK AND C. G. KING 1936 Factors influencing the vitamin C content of vegetables. Am. J. Pub. Health, vol. 26, p. 905.

THE INFLUENCE OF SPECIFIC MINERAL DEFICIENCIES ON THE GROWTH OF BODY AND ORGANS OF THE RAT [1]

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ONE FIGURE

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Previous studies (Swanson and Smith, '32, '34, '36) have shown that strict limitation of inorganic salts in the diet of the albino rat results not only in cessation of the increase in body weight, but also in changes in the growth of specific organs. Thus after 60 to 90 days the heart and spleen weigh less than expected for a normal animal of the same body weight, and the kidney and adrenal glands are heavier than usual. The experimental ration used in these studies was notably poor in calcium, sodium, magnesium and chlorine, whereas the relative proportions of phosphorus and of potassium were high (Smith and Smith, '34). In the present study, the influence of individual elements and groups of elements upon the somatic growth and development of certain organs was determined.

Inasmuch as the salt mixture of Osborne and Mendel ('17) was found to be satisfactory for normal development when

[1] The data forming the basis of this paper were taken from a dissertation submitted by Ercel S. Eppright in partial fulfillment of the requirement for the degree of doctor of philosophy, Yale University, 1936.

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incorporated in the basic salt-poor experimental diet, the amounts of the individual inorganic elements included in the various modifications of the basal diet were patterned after it, due account being taken of the consumption of the energy-yielding portion of the ration. Osborne and Mendel ('18) studied the individual elements in a somewhat similar manner, but the subsequent progress in nutrition justifies a reinvestigation of the problem.

PLAN OF STUDY

Male albino rats from the Connecticut Agricultural Experiment Station strain, weighing 45 gm. ±4 at weaning (21 days old) were allowed free access to an adequate stock ration consisting of modified calf meal [2] (Maynard, '30) and paste food,[3] supplemented daily with lettuce and yeast. If they attained a weight of 120 ± 4 when they were 34 days old ±2, they were selected for the experiment.

The basal diet used in all the groups except one (group V) consisted per 100 gm. of

	gm.
Casein[4]	18
Hydrogenated fat[5]	27
Dextrin[6]	55

Vitamins were provided separately each day by 200 mg. dried yeast, 5 drops cod liver oil, 1 ml. of alcoholic extract of wheat germ, and 2 drops of wheat germ oil.

In the basal diet of group V, low-ash lactalbumin[7] replaced the casein and 160 mg. of liver extract,[8] 1 ml. of alcoholic extract of wheat germ, and 5 drops of cod liver oil comprised the vitamin adjuvants.

[1] Modified by the addition of cod liver oil, 3%.

[2] Whole milk powder, 25%; casein, 25%; wheat germ, 20%; lard, 30%.

[4] Described by Swanson and Smith ('32).

[5] Crisco.

[6] White, commercial.

[7] Dry basis, total nitrogen, 14.2%; calcium, none; phosphorus, 0.14%. The Dry Milk Co.

[8] Eli Lilly liver extract no. 343.

The appetite of the low-salt rat is poorer than that of the normal rat. Its daily food consumption has been determined in previous investigations (Brooke and Smith, '33; Clarke and Smith, '35). In order to eliminate the influence of variations in intake of energy, protein and vitamins of the diets, all animals of the present study were given each day the amount of food indicated by the average intake of the large number of low-salt rats previously studied, according to the following schedule:

Period	*Grams of basal diet given per rat per day*
1– 7 days	7.1
8–14 days	6.4
15–35 days	5.7
35–60 days	5.6

The daily quantities of inorganic elements were for the most part based upon 408 mg. of Osborne and Mendel ('17) salt mixture, since this amount represents the average daily consumption of normal male rats eating ad libitum an adequate synthetic diet containing 4% of the salt mixture, a quantity long considered adequate. Formulas were calculated for each diet and for each period in such a way that the desired amount of inorganic adjuvant was offered daily to each rat together with the desired amount of basal ration. Table 1 shows the various groups, the mineral supplements, and the amounts of the different elements given daily together with the abbreviations by which the groups are hereafter designated.

The method insured a fair degree of uniformity in food consumption throughout the groups. As would be expected, however, some animals did not conform exactly to the schedule. These, when possible, were fed by hand. The animals were given redistilled water, and their caging and care followed closely the suggestions of Smith, Cowgill and Croll ('25).

Since most of the changes produced by the restriction of dietary salts are evident after 60 days on the diet, the present experiments were terminated at that time. The animals were

weighed at 4-day intervals just preceding their regular daily feeding. The weights were averaged and treated statistically where possible. Under ether anesthesia the animals were bled from the abdominal aorta and the organs quickly removed, trimmed, blotted, placed in a covered tared bottle, and weighed as quickly as possible.

TABLE 1

Inorganic elements added to diets

GROUP	MINERAL SUPPLEMENT		MILLIGRAMS SUPPLIED DAILY					
	Salt mixture	Milligrams given daily	Ca	Mg	Na	K	Cl	P
I	O and M[1]	408	50.3	7.2	15.4	80.0	21.9	40.9
II	O and M[2] — NaCl + K	408	99.3	7.2	1.7	7.2	3.9	44.5
III	Ca + P + Mg[3]	176	50.3	7.2	1.4	7.0	3.9	40.9
IV	Ca + P[4]	152	50.3	0.2	1.4	7.0	3.9	40.9
V	$CaCO_3$ (lact.)	126	50.3	?	1.1	5.0	...	4.0(?)
VI	$CaCO_3$ (restrict.)	25.6	10.2	0.2	1.4	7.0	3.9	14.6
VII	NaCl + K[5]	242	0.3	0.2	15.4	63.0	24.7	14.6
VIII	NaCl	35.6	0.3	0.2	15.4	7.0	24.7	14.6
IX	Low-salt	...	0.3	0.2	1.4	7.0	3.9	14.6

[1] Prepared as described in J. Biol. Chem., vol. 32, p. 369 ('17).

[2] Essentially Osborne and Mendel salt mixture with sodium and potassium carbonates and hydrochloric acid omitted; calcium carbonate was increased to 238.1 gm.

[3] 152 gm. calcium-phosphorus salt mixture; 24 gm. magnesium carbonate ($3\ MgCO_3 . Mg(OH_2) . 3\ H_2O$).

[4] Calcium carbonate, 404.4 gm.; phosphoric acid, 309.6 gm. of 85% (180.9 ml. of sp.g. 1.71).

[5] Potassium citrate ($K_3C_6H_5O_7H_2O$), 207.8 gm.; sodium chloride, 35.6 gm.

RESULTS

As previously stated, an effort was made to limit the intake of all groups to the amount of energy-yielding and indispensable organic nutrients consumed by the low-salt animals (group IX); these on the average eat approximately half the total amount of food consumed by rats allowed free access to an adequate synthetic diet. The influence of the different salts on the acceptance of the diet was striking. Sodium chloride failed to render the low-salt ration more attractive. Contrary

to expectations, according to the theory of Bunge, reinforcement of the sodium chloride supplement with potassium did not increase the appetite. On the other hand, when calcium was added to the casein diet, either alone, as the carbonate, or with phosphorus, prompt and complete food consumption resulted. Either 10 or 50 mg. of calcium proved sufficient under these experimental conditions. At least a small amount

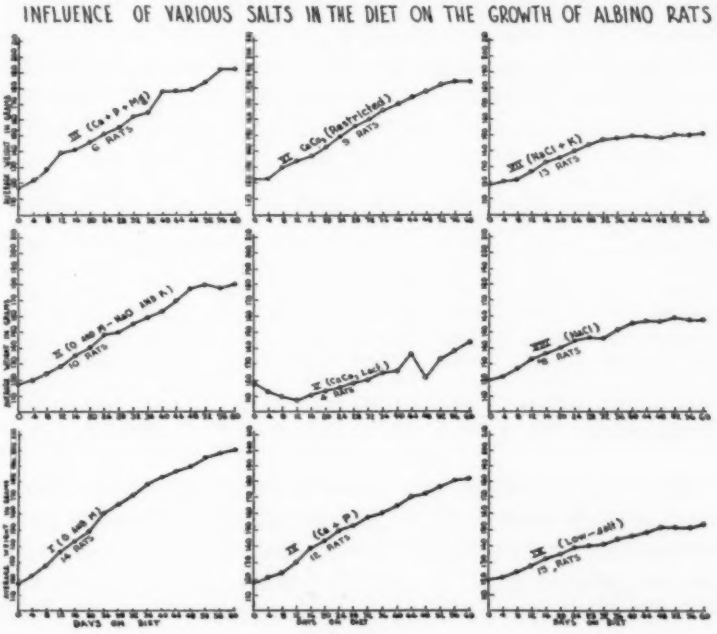

Figure 1

of phosphorus was apparently essential, for when 50 mg. of calcium were ingested daily in the phosphorus-poor lactalbumin low-salt diet, the rats consumed their food less readily.

Growth of animals. Figure 1 shows the average growth curves of the groups. When the total ash constituents of the diet were reduced to 25 mg. per day, growth was markedly retarded, an observation which corroborates that of previous

investigations (Swanson and Smith, '34). Eight days of such rigid restrictions were sufficient to produce a statistically significant difference in body weight as compared with animals similarly limited as to calories and essential protein adjuvants but ingesting daily 408 mg. of the Osborne and Mendel salt mixture (group I). This difference increased until at the end of 60 days the low-salt rats (group IX) had made on the average a gain only 41% of that of group I.

TABLE 2

Weights of organs on low-salt and replacement diets—averages in grams

GROUPS	I	II	III	IV	VI	VII	VIII	IX
ORGAN	O and M	O and M − NaCl + K	Ca + P + Mg	Ca + P	$CaCO_3$ (restrict.)	NaCl + K	NaCl	Low-salt
Liver	5.720	5.179	5.221	5.014	5.044	5.837	5.876	5.543
Kidney	1.275	1.269	1.226	1.328	1.315	1.412	1.723	1.338
Lung	1.067	0.879	0.862	0.872	0.943	0.835	0.962	0.820
Spleen	0.347	0.306	0.310	0.328	0.312	0.344	0.294	0.279
Heart	0.656	0.605	0.615	0.572	0.595	0.621	0.664	0.597
Thymus	0.175	0.184	0.179	0.165	....	0.089	....	0.101
Thyroid	0.014	0.015	0.013	0.015	....	0.014	0.013	0.013
Pituitary	0.0057	0.0051	0.0054	0.0054	....	0.0053	0.0055	0.0054
Body weight	200	180	192	180	184	153	155	152
Number of rats	12	10	6	12	9	12	8	13

The addition of 35 mg. of sodium chloride to the casein low-salt diet (group VIII) did not produce growth significantly greater than that of the animals given the unsupplemented low-salt diet. The addition of 65 mg. of potassium as the citrate to the sodium chloride supplement (group VII) produced such variable results that the average can scarcely be taken as representative of the group; the maximum weight reached after 60 days on this diet was much less than that of group I (Osborne-Mendel salts), and the averages at the various weighing periods were at no time significantly greater than those of the low-salt rats (group IX). Conversely, as was shown by group II, maximum growth was impossible in the absence of added sodium, potassium and chlorine. Evidently, 1.4 mg. sodium and 7.0 mg. potassium, the quantities

of each found in the daily allowance of the basal low-salt diet, are suboptimal for growth. Nevertheless, when other ions were withheld from the diet, the addition of these elements in the quantities normally consumed by rats had no favorable influence on growth.

When calcium carbonate (50 mg. calcium) was added to the lactalbumin low-salt diet as herein described, growth was not appreciably increased. However, when as little as 10 mg. of calcium (as the carbonate) was added to the casein low-salt diet good growth ensued. In all of the groups given diets supplemented with calcium and phosphorus the rate of growth was greatly improved. After 16 days on the diet of group IV

TABLE 3

Summary of organ weights expressed in per cent of body weight—averages

	GROUPS	KIDNEY	LIVER	HEART	LUNG	SPLEEN	THYMUS
I	O and M	0.64	2.88	0.33	0.53	0.17	0.086
II	O and M — NaCl + K	0.70	2.90	0.33	0.49	0.17	0.099
III	Ca + P + Mg	0.63	2.72	0.32	0.44	0.16	0.092
IV	Ca + P	0.73	2.77	0.31	0.48	0.18	0.082
VII	NaCl + K	0.74	3.94	0.40	0.55	0.19	0.078
VIII	NaCl	1.08	3.79	0.42	0.62	0.19	....
IX	Low-salt	0.89	3.68	0.39	0.54	0.18	0.067

(calcium and phosphorus), the animals were significantly larger than the low-salt animals, but after 24 days they were significantly smaller than the animals given the complete salt mixture. This intermediate position of the growth curve of group IV between group I and group IX was maintained until the end of the experiment, and ultimately it appeared that calcium and phosphorus alone were jointly capable of supporting approximately 75% of the growth made on the complete salt mixture under these experimental conditions.

In group III, which differed from group IV only in the addition of magnesium, growth was slightly better than in group IV; however, the limited number of rats in the group prevents conclusions regarding the beneficial nature of this quantity of magnesium. While the magnesium requirement

of the albino rat has not been determined, it is likely from the present data that the amount of this element (0.018 milli-equivalents per day) in the basal low-salt diet is insufficient for growth. Osborne and Mendel ('18) observed fair growth on approximately 0.05 milli-equivalent of magnesium per day, whereas Leroy ('26) observed nutritional failure on approximately 0.004 milli-equivalents per day.

Growth of organs. The low-salt diet alters the growth of certain organs; it is stimulating to some while it retards the development of others. After 90 days on the diet the kidneys and adrenal glands hypertrophy, whereas the spleen and heart regress (Swanson and Smith, '36). In the present study the influence of the low-salt diet on the size of these and other organs has been reinvestigated and, in addition, the effects produced by the replacement of specific salts and groups of salts have been observed. Inasmuch as the growth of some organs bears a close correlation to growth of the body as a whole, while others do not, the results here obtained were reported both as absolute fresh weight and in relation to body weight.

The livers of the low-salt rats were grossly not significantly different in size from the livers of rats given all of the salts. The replacement of calcium and phosphorus in the diet (as in groups II and IV) resulted in significantly smaller livers than those of group I. With the replacement of sodium chloride or sodium chloride plus potassium, the average gross weights of the liver at 60 days were as large as those of animals ingesting all the salts.

The growth of the liver bears a fairly close correlation to the growth of the body as a whole, the coefficient as given by Donaldson ('24) being 0.83. In groups II and IV, although the livers were grossly small, the proportion of hepatic tissue to body weight was practically normal. However, in groups VII, VIII and IX the rate of increase in hepatic tissue was greater than that of the body as a whole.

The actual weights of the kidneys were extremely variable in all groups except group I. In the low-salt and most of the

replacement groups, these organs were somewhat larger than normal, but the differences were not statistically significant. In group VIII (NaCl) in which there was unmistakable renal and bladder involvement, some of the kidneys were extremely large, weighing as much as 3.11 gm. per pair. In the kidneys of group IV, which were slightly but not significantly large, histological examination revealed numerous calcium casts. This cast formation which was similar to that induced by parathormone administration was apparently induced by normal amounts of calcium and phosphorus but in the absence of other salts in the diet.[9]

In groups VII (NaCl plus K), VIII (NaCl) and IX (low-salt), the proportion of renal weight to body weight was markedly disturbed. The animals of these groups, after 60 days on the experimental diet, possessed renal tissue 15.6%, 69.0% and 36.8%, respectively, greater per gram of body weight than the average of the animals of group I (Osborne-Mendel salts). The kidneys of rats thus fed continued to grow despite the cessation of body growth.

In all of the groups in which no sodium, potassium, or chloride were added to the basal diet (i.e., groups II, III, IV and IX), the hearts were grossly smaller on the average than those of group I (Osborne-Mendel salts). With these elements supplied (groups VII and VIII) the hearts were not appreciably different in size from those of group I. In all the groups amenable to statistical treatment, these observations were valid. Sodium chloride, with or without potassium, was sufficient to stimulate growth of the heart comparable to that of animals given all the salts and was capable of sustaining such growth independent of increase in body weight.

The only significant difference with respect to the size of the spleens was found in group IX, the low-salt group; the average gross weight of the spleens of this group was 19.6% less than that of group I (Osborne-Mendel salts). In relation to body weight, the splenic tissue of the low-salt rats was not

[9] We are indebted to Dr. H. A. Weiner of the department of pathology for interpretation of the renal changes.

diminished. Throughout the groups the ratio of splenic tissue to body weight was strikingly constant.

In the low-salt and all of the replacement groups except group VIII (NaCl), the lungs were significantly smaller than those of group I (Osborne-Mendel salts). Related to body weight the pulmonary tissue of the sodium chloride supplemented group VIII (NaCl) was 16.7% greater than that of group I. The pulmonary tissue of the other groups retained an approximately uniform proportionality to body weight.

The thymus glands of albino rats maintained for 60 days on the low-salt diet (group IX) were significantly smaller than those of animals given all the salts (group I). The addition of sodium, potassium and chlorine (group VII) contributed nothing toward the growth of the thymus gland; in fact the thymus glands in this group were small and different in form, color and general appearance from the glands of the animals in better condition. In the rats consuming the rations with calcium and phosphorus, the thymus glands were like those of the animals in group I in size; the presence or absence of sodium and/or potassium was without influence. Despite the stunted somatic growth, the thymus tissue per unit of body weight of the low-salt rats (group IX) was less than that of group I (Osborne-Mendel salts) or of group IV (Ca plus P). This stimulation of growth of the thymus gland by calcium and phosphorus in the diet is of interest in connection with the suggestions that this organ is related to calcium metabolism (Zwarenstein, '34).

None of the modifications of the mineral content of the diet produced significant changes in the pituitary glands when fresh weight was taken as the criterion.

While the thyroid glands of the rats of group II and group IV, both of which were supplemented with calcium, weighed slightly more than those of the other groups, the enlargement was not significant. In the present series no significant changes in the thyroid glands were observed.

Because of the frequently suggested relationship between the adrenal glands and mineral metabolism a special study

was made of these organs in a larger series of replacement experiments than herein reported. The data thus secured will be presented in a subsequent paper.

Other changes in tissues. Usually the hair of rats given the casein low-salt diet begins to fall until, after a month on the ration, the abdominal area and legs may become almost bare. The remainder of the coat is unkempt and lusterless. The replacement of sodium chloride (group VIII) or sodium chloride plus potassium (group VII) in the diet did not prevent the loss of hair. However, when the diet included calcium, and phosphorus in addition to that in the casein of the basal ration, loss of hair did not occur. The importance of both elements in this connection is emphasized by the observation that calcium unsupplemented with phosphorus did not prevent loss of hair.

Excitability. Albino rats deprived of salts were in a state of hyperexcitability not nullified by any of the salts given separately. A marked disproportion either of calcium or of potassium in the diet augmented the condition. Toward the end of the experimental period of 60 days, members of group VII (sodium chloride plus potassium replaced) were frequently found in a state of tetany.

Hyperemia. The five rats of group V, which were given daily doses of 126 mg. of calcium carbonate as the only inorganic supplement to the lactalbumin low-salt diet, after 24 to 30 days on the diet, developed a redness of the ears which persisted for about 7 days and then practically disappeared, leaving the ears scabby and pale. These evidences of capillary disturbances were apparent in other parts of the body. The condition was strikingly similar to that described by Kruse, Orent and McCollum ('32) in rats given a diet very low in magnesium. The animals were somewhat excitable, but the extreme nervousness and spasticity described by these investigators were observed in only two members of the group.

In only one other group was this same condition observed even to a limited extent; four of the twelve rats given calcium

and phosphorus (group IV) developed hyperemia on the ears, and scabby areas found in the skin suggested capillary disturbances similar to those occurring in the ears.

Sooner or later during the period of low-salt feeding, the animals show dried blood around the nose. This condition is not correlated with lesions in the lungs but seems to be associated with capillary fragility in the nasal or tracheal mucosa. It was particularly noticeable that this condition was absent in the rats of group IV, given calcium and phosphorus.

The amount of magnesium ingested daily by the animals of group V is not known; in group IV, as was previously stated, 0.2 mg. comprised the daily intake. The diets used by Kruse, Orent and McCollum ('32) contained only 1.8 parts magnesium per million. The fact that the hyperemia occurred in only a limited number of group IV may indicate that 0.2 mg. is near the minimum requirement. Moreover, the fact that the hyperemia did not occur in groups VI, VII and IX which were likewise poor in magnesium but were also poor in calcium may provide further evidence that the calcium-magnesium ratio plays an important role in the etiology of the syndrome.

SUMMARY

With the intake of food calories limited to approximately one-half the usual consumption, calcium and phosphorus are the most effective mineral supplements used in this study from the point of view of increase in body weight, maintenance of size of thymus, and general nutritive well-being. Although sodium and potassium, given separately or together, fail to promote growth in the absence of the remainder of the elements of the Osborne and Mendel salt mixture, their presence is required to support the maximum development possible on the given energy and protein allowance. In the groups consuming rations without calcium but with sodium and/or potassium, the ratios of both heart weight and liver weight to body weight were elevated.

LITERATURE CITED

BROOKE, R. O. AND A. H. SMITH 1933 Inorganic salts in nutrition. VI. The mineral metabolism of rats receiving a diet low in inorganic constituents. J. Biol. Chem., vol. 100, p. 105.

CLARKE, M. F. AND A. H. SMITH 1935 Effect of a diet poor in salts upon the growth and composition of the incisors of the rat. Am. J. Physiol., vol. 112, p. 286.

DONALDSON, H. H. 1924 The rat. The Wistar Institute of Anatomy and Biology. Philadelphia.

KRUSE, H. D., E. R. ORENT AND E. V. MCCOLLUM 1932 Studies on magnesium deficiency in animals. I. Symptomatology resulting from magnesium deprivation. J. Biol. Chem., vol. 96, p. 519.

LEROY, J. 1926 Necessite du magnesium pour les croissance de la souris. Compt. rend. soc. biol., vol. 94, p. 431.

MAYNARD, L. A. 1930 A diet for stock rats. Science, vol. 71, p. 192.

OSBORNE, T. B. AND L. B. MENDEL 1917 The use of the soy bean as food. J. Biol. Chem., vol. 32, p. 369.

———— 1918 The inorganic elements in nutrition. J. Biol. Chem., vol. 34, p. 131.

SMITH, A. H., G. R. COWGILL AND H. H. CROLL 1925 A note on technique for studying vitamin B. J. Biol. Chem., vol. 66, p. 15.

SMITH, A. H. AND P. K. SMITH 1934 Inorganic salts in nutrition. X. Electrolyte balance in the serum of rats receiving a diet deficient in inorganic constituents. J. Biol. Chem., vol. 107, p. 681.

SWANSON, P. P. AND A. H. SMITH 1932 Inorganic salts in nutrition. IV. Changes induced in the blood by a ration deficient in inorganic constituents. J. Biol. Chem., vol. 98, p. 479.

———— 1934 Inorganic salts in nutrition. IX. Correlation between suppressed growth and the development of polycythemia induced by feeding a ration poor in salts. J. Nutrition, vol. 8, p. 659.

———— 1936 Effect of restriction of inorganic salts in the diet on organ growth. Am. J. Physiol., vol. 116, p. 516.

ZWARENSTEIN, H. 1934 The endocrine glands and calcium metabolism. Biol. Rev., vol. 9, p. 299.

GROWTH ON HISTIDINE AND LYSINE ADMINISTERED BY SUBCUTANEOUS OR INTRAPERITONEAL INJECTION [1]

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TWO FIGURES

(Received for publication December 23, 1936)

It is well established that the dietary lack of certain amino acids results in cessation of growth, presumably because the body requires them for tissue building or for other normal functions and is unable to supply them by synthesis. If this thesis is correct, growth should again occur when the deficiency is met; the mode of administering the required amino acid should enter into the consideration only insofar as it might influence the rate of supply to the tissues. In other words, the same qualitative response in growth should be obtained whether the amino acid is given by mouth or otherwise; on the other hand, quantitative differences might reasonably be expected unless the methods of administration insured the same continuity and uniformity in distribution of the amino acid.

Alcock ('36) has recently questioned the assumption that preformed amino acids are used for tissue synthesis. He has based his doubt largely on his earlier finding ('34) that tryptophane administered subcutaneously for 15 days to two

[1] The experimental data are taken from a dissertation submitted by Ralph M. Conrad in June, 1936, in partial fulfillment of the requirements for the degree of doctor of philosophy in biochemistry in the Graduate College of the State University of Iowa. The data were presented in abstract before the Iowa Academy of Science at Iowa City, Iowa, April 3, 1936.

animals on a tryptophane-deficient diet did not improve their nutritive condition. Unfortunately, however, his experimental data do not seem adequate to justify his far-reaching assumption ('36) or even to refute prior contrary evidence.

Thus far the effectiveness of administering essential amino acids by injection has been tested only on tryptophane. The earliest such study was made by Jackson ('27) on two animals, each of which was given 25 mg. of tryptophane once daily over a 12-day period. The data show that although the animals did not grow, neither did they undergo as rapid loss in weight as the control animal injected with water. Shichiri and Sakata ('33) gave extremely small doses of tryptophane (1 mg. every other day, followed by 1, 2 and 4 mg. daily) over a long period of time. The three animals thus treated lived until the termination of the study, approximately 120 days after they had been shifted from a casein diet to a deficient diet thus supplemented. All of the animals lost considerably less weight (by 40 to 50 gm.) than the three controls which received no injections. Furthermore, all of the latter died approximately 80 days after the deficient regimen was begun. In their earlier study, du Vigneaud, Sealock and Van Etten ('32) injected a single animal with 10 mg. of tryptophane daily, distributed in four equal doses, for a period of 14 days. They obtained a striking growth response which they were able to duplicate in a second animal on 12 mg. of acetyl-l-tryptophane, similarly administered. The recent and more extensive observations of du Vigneaud, Sealock and Van Etten ('35–'36) fully substantiate their earlier conclusion that tryptophane can be utilized when injected subcutaneously.

Alcock compared the growth of but two animals, each injected subcutaneously with 20 mg. of tryptophane daily in one dose for a period of only 15 days, with the growth of two animals receiving no supplements. Careful measurement of the growth curves indicates that the injected animals actually did grow slightly better than the controls. Furthermore it should be noted that both controls grew slightly during the 15 days, whereas on a preliminary test of the same diet, four

out of six rats lost weight during a 20-day period and five of the six showed loss when the period was extended to 40 days. The differences in growth of the two experimental animals and the two controls cannot in themselves be considered adequately positive, but we cannot agree with Alcock ('36) that the result was 'definitely negative.' In our opinion the data suggest that the test period was not sufficiently long to demonstrate utilization of tryptophane thus administered. Certainly the results were not sufficiently striking in the face of contrary evidence to serve as one of the premises upon which to base a revolutionary hypothesis.

Two significant variations are to be noted in the several studies. The experimental animals of du Vigneaud, Sealock and Van Etten weighed much less (57 to 98 gm.), with but two exceptions (115 and 146 gm.), than any of the rats employed in the other studies (130 to 187 gm.); furthermore, their animals were placed upon a deficient diet for several days before the injections of tryptophane were begun. This procedure is especially important in studies lasting for such short periods as 12 to 15 days. Rats transferred from a stock diet to a synthetic diet, or from a better synthetic diet to a poorer one, frequently lose weight for several days before actually gaining, even though the new diet is adequate. On the other hand, rats which have subsisted for a time on a diet deficient in an essential amino acid usually show prompt growth response when the missing component is supplied.

Because the theoretical implications in these studies involve other essential amino acids, we undertook to test the growth-promoting ability of lysine and histidine when supplied either by subcutaneous or by intraperitoneal injection to rats on diets deficient in one or the other. The data recorded below confirm the view that essential amino acids can be utilized, whether fed or injected.

EXPERIMENTAL

The eighteen young rats used in the histidine studies were all of the same age and weighed between 33 and 56 gm. They

were housed in individual false-bottomed cages and were fed the histidine-deficient diet and vitamin supplements as outlined in table 1. Food consumption and body weights were recorded every 4 days. After an 8-day preliminary period on the deficient diet, all but two animals were given 37 mg. of histidine daily. The histidine was administered as the monohydrochloride in one of three ways: 1) by subcutaneous injection, 2) by intraperitoneal injection, or 3) by incorporation in

TABLE 1

Composition of diets

	HISTIDINE-DEFICIENT DIET [1]	LYSINE-DEFICIENT DIET [1]
	%	%
Histidine-deficient casein hydrolysate [2]	14.5	...
Zein [3]	...	19.5
Tryptophane	0.2	0.2
Cystine	0.3	0.2
Histidine monohydrochloride	...	0.1
Starch	39.5	34.5
Sucrose	15.0	15.0
Hydrogenated cottonseed oil [4]	19.0	19.0
Cod liver oil	5.0	5.0
Salt mixture (Hawk and Oser, '31)	4.5	4.5
Agar	2.0	2.0
	100.0	100.0

[1] The diets were fed ad libitum. Two 100 mg. vitamin B complex tablets (Harris) were supplied separately daily. Distilled water was always available.

[2] Prepared as directed by Conrad and Berg ('37).

[3] For preparation, see Berg ('36).

[4] Crisco.

the vitamin pills. Of the first group, three rats received the daily supplement in two equal doses 12 hours apart and three were given it in a single dose; in the other groups, three animals received two doses and two a single dose daily. The hydrochloric acid was always neutralized by the addition of an equivalent amount of sodium bicarbonate. The solutions for injection were of such strength that the daily supplement was contained in 1 cc. The solutions were sterilized by autoclaving and all reasonable precautions were taken to prevent

infections. The injections were made in different places to prevent unnecessary irritation. The administration of histidine was continued for 32 days; the experiment was terminated 8 days later.

In the lysine studies the same technics were employed. The twenty rats were all of the same age and showed essentially the same range in initial weights (37 to 58 gm.) as those in the histidine series. The composition of the lysine-deficient diet is recorded in table 1. After an 8-day preliminary period on this diet all but two rats received 50 mg. of lysine daily as the dihydrochloride for a period of 32 days. Again exactly enough sodium bicarbonate was added to convert the hydrochloride to the free amino acid. Each of the three types of supplementation was given to six animals, three of which received the lysine in two daily doses and three in one. The study was terminated 12 days after the lysine supplements were discontinued.

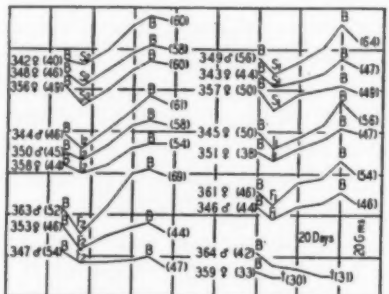
DISCUSSION OF RESULTS

Individual growth curves are presented in figures 1 and 2. In every instance the animals on the histidine-deficient diet lost weight. When the histidine administrations were begun, marked improvement was noted which continued until the supplements were omitted. In every instance both the subcutaneous and intraperitoneal injections induced growth. The data show very clearly that the histidine thus administered was utilized. In fact, growth on histidine supplied by injections compared much more favorably with growth on histidine fed separately than we had anticipated.

These same statements can be made of the lysine series of animals. In every instance differences between growth on the supplemented and unsupplemented zein diets were striking. This was true whether the lysine was injected or given by mouth.

A careful comparison of the several growth curves of the rats receiving two doses of lysine or histidine with those of animals receiving only one slightly favors the former. Individual variations in growth response (table 2) were wide,

however. The use of more animals or an extension of time beyond the 32 days would undoubtedly have minimized these irregularities and made distinctions sharper.



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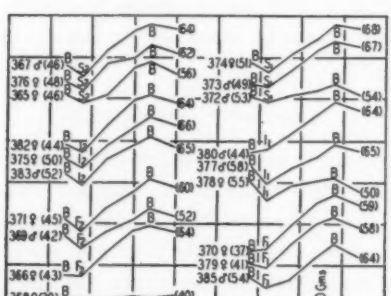

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Figs. 1 and 2 Initial and final weights of the rats are given in parentheses. B represents the deficient basal diet alone. S indicates the supplementation of B by subcutaneous injection of the required amino acid; I, supplementation by intraperitoneal injection; and F, by separate feeding in the vitamin pill. Administration of the supplement in one dose daily is represented by the subscript 1; in two doses daily by subscript 2. The daggers indicate death.

The results which we have obtained are not in harmony with Alcock's ('34) interpretation that injected amino acids do not support growth. They constitute, instead, a refutation of one of his arguments against current concepts of protein synthesis in vivo ('36). In our opinion Alcock failed to ob-

TABLE 2

Average daily growth and food consumption during the 32 day period

TABLE 2

Average daily growth and food consumption during the 32-day period of amino acid administration

RAT NO. AND SEX	AVERAGE DAILY		TYPE AND FREQUENCY OF DAILY SUPPLEMENTATION	RAT NO. AND SEX	AVERAGE DAILY		TYPE AND FREQUENCY OF DAILY SUPPLEMENTATION
	Gain in weight	Food consumption			Gain in weight	Food consumption	
	gm.	*gm.*			*gm.*	*gm.*	
Histidine series [1]							
342♀	0.75	3.9	Subcutaneously (twice)	343♀	0.41	3.4	Subcutaneously (once)
348♀	0.66	3.3	Subcutaneously (twice)	349♂	0.75	3.5	Subcutaneously (once)
356♀	0.69	3.4	Subcutaneously (twice)	357♀	0.38	3.2	Subcutaneously (once)
344♂	0.81	3.9	Intraperitoneally (twice)	345♀	0.69	3.5	Intraperitoneally (once)
350♂	0.59	3.0	Intraperitoneally (twice)	351♀	0.47	3.3	Intraperitoneally (once)
358♀	0.44	3.6	Intraperitoneally (twice)				
363♂	0.97	4.0	Per os (twice)	361♀	0.66	3.3	Per os (once)
353♀	0.41	3.6	Per os (twice)	346♂	0.41	3.3	Per os (once)
347♂	0.09	3.2	Per os (twice)				
364♂	—0.18	2.3	None (died on the 28th day of the period)				
Lysine series [2]							
367♂	0.70	4.1	Subcutaneously (twice)	374♀	0.72	4.0	Subcutaneously (once)
376♀	0.69	4.3	Subcutaneously (twice)	373♂	0.69	4.2	Subcutaneously (once)
365♀	0.63	4.1	Subcutaneously (twice)	372♂	0.28	3.2	Subcutaneously (once)
382♀	0.88	4.2	Intraperitoneally (twice)	380♂	0.69	3.8	Intraperitoneally (once)
375♀	0.75	3.9	Intraperitoneally (twice)	377♂	0.56	4.1	Intraperitoneally (once)
383♂	0.72	3.9	Intraperitoneally (twice)	378♀	0.31	3.7	Intraperitoneally (once)
371♀	0.84	3.8	Per os (twice)	370♀	0.81	4.2	Per os (once)
366♀	0.75	4.1	Per os (twice)	379♀	0.75	3.6	Per os (once)
369♂	0.63	3.6	Per os (twice)	385♂	0.66	4.0	Per os (once)
384♀	0.06	2.6	None				
368♀	0.03	3.0	None				

[1] During the initial 8 days, animals in the histidine series lost 0.71 gm. per day on the average and ingested 3.1 gm. of food per day; during the 8-day after period corresponding average figures were 0.51 gm. and 3.3 gm., respectively, for all rats which had previously received histidine.

[2] Losses for the animals of the lysine series during the initial 8 days averaged 0.51 gm. per day; during the final 12 days 0.36 gm. Average daily food consumption figures for the two respective periods were 3.5 gm. and 3.9 gm.

tain definitely positive results with tryptophane largely because his test period, as conducted, was too short. Had the injections followed a period of deficiency, as in the case of the present histidine and lysine studies, a more immediate response might have occurred. Incidentally, feeding an essential amino acid in an intimate mixture with other dietary components (Alcock, '34) cannot be considered a suitable control for a regimen in which the essential amino acid is administered by injection in one or two doses. The two regimens are not analogous. We have partially overcome this objection in the present studies (as indeed du Vigneaud, Sealock and Van Etten did in theirs, '36) by incorporating the amino acid in the separate vitamin B supplement. This procedure is not ideal, but it permits a much fairer comparison and is practical.

In our several studies on tryptophane, lysine and histidine metabolism, we have invariably obtained better growth by incorporating these amino acids in the otherwise deficient diet than by feeding them separately in the vitamin pills. In fact, significant variations in growth are induced, even when the same quantity of tryptophane is fed separately, by altering the frequence of dosage (Berg and Rose, '29). These observations are reasonable and in accord with the accepted theory that an essential amino acid cannot be utilized efficiently for tissue synthesis unless the other amino acids needed in the construction are also present in adequate amounts. The administration of one or two relatively massive doses each day would naturally provide a great excess at one time and a shortage at others.

CONCLUSIONS

Both histidine and lysine can be utilized effectively for growth when administered to rats either by subcutaneous or by intraperitoneal injection. The rate of growth of such animals compares favorably with that of other animals fed the same amino acids separately. These observations are entirely in accord with currently accepted theories of protein metabolism.

LITERATURE CITED

ALCOCK, R. S. 1934 Tryptophane deficiency. Biochem. J., vol. 28, p. 1721.

———— 1936 The synthesis of proteins in vivo. Physiol. Rev., vol. 16, p. 1.

BERG, C. P. 1936 The availability of *d*(—)-lysine for growth. J. Nutrition, vol. 12, p. 671.

BERG, C. P. AND W. C. ROSE 1929 Tryptophane and growth. I. Growth upon a tryptophane-deficient basal diet supplemented at varying intervals by the separate feeding of tryptophane. J. Biol. Chem., vol. 82, p. 479.

CONRAD, R. M. AND C. P. BERG 1937 The optical inversion of *d*-histidine in the animal body. J. Biol. Chem., vol. 117, p. 351.

HAWK, P. B. AND B. L. OSER 1931 A modification of the Osborne-Mendel salt mixture. Science, vol. 74, p. 369.

JACKSON, R. W. 1927 Indole derivatives in connection with a diet deficient in tryptophane. J. Biol. Chem., vol. 73, p. 523.

SHICHIRI, G. AND H. SAKATA 1933 XIV. Mitteilung: Wird das Tryptophan bei subcutaner Darreichung nicht zur Ernahrung verwendet? Z. physiol. Chem., Bd. 214, S. 14.

DU VIGNEAUD, V., R. R. SEALOCK AND C. VAN ETTEN 1932 The availability of *d*-tryptophane and its acetyl derivative to the animal body. J. Biol. Chem., vol. 98, p. 565.

———— 1935–1936 The question of the utilization of tryptophane administered subcutaneously. J. Biol. Chem., vol. 112, p. 451.

ON THE CLAIM FOR A NEW ESSENTIAL DIETARY FACTOR IN MAMMALIAN LIVER[1]

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TWO FIGURES

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Elvehjem and co-workers ('35, '36 a, '36 b) and Koehn and Elvehjem ('36) have reported a new dietary factor from liver which they find to be necessary in addition to vitamins B(B_1) flavin, B_4, B_6 and 'B_2' [which, it would seem, is identical with the 'filtrate factor' of Lepkovsky and Jukes ('35, '36 a, '36 b) and Lepkovsky, Jukes and Krause ('36)] for growth of rats.

We wish to report certain results which we have obtained with the alcohol-ether precipitate from air-dried fresh hog liver, following the procedure described by these authors. From the supernatant liquid we prepared an adsorbate with the resulting filtrate. A water extract of the precipitate showed no fluorescence when tested by the 'black light' (Supplee and others, '36), but the supernatant liquid was highly fluorescent.

EXPERIMENTAL

Two types of basal diets have been used in this laboratory in tests for flavin and vitamin B_6 (Halliday and Evans, '37). Diet 787-B has the following composition: casein 18%, salts 4%, filtered butter fat 8%, cod liver oil 2% and cornstarch

[1] Aided by grants from the Board of Research and the College of Agriculture of the University of California, and the Rockefeller Foundation of New York. The following materials were generously contributed: Cod liver oil by E. R. Squibb and Sons; vitamin B_1 concentrate by Merck & Co., through the courtesy of Dr. R. E. Gruber; Lactoflavin by the Vitab Products Company, through the courtesy of Mr. G. F. Siemers; fresh hog liver by H. Moffat Company, San Francisco, through the courtesy of Mr. F. M. Kleppe.

68%. In diet 793 the butter fat is reduced to 3% and 73% sucrose is included in the place of cornstarch. The casein is extracted with boiling 95% alcohol until no fluorescence can be detected when the alcohol extract is examined by a 'black light' (four extractions). This procedure is followed by two extractions with cold 60% alcohol and one with cold 95% alcohol.

Twenty-one-day-old female rats weighing 40 to 50 gm. were given the basal diet, supplemented by 200 γ of a potent vitamin B concentrate until the weight was stationary and mild dermatitis developed. This required about 4 to 5 weeks with diet 787-B, and 3 to 4 weeks with diet 793. With the latter diet, rats receiving B or B and flavin, with or without a supplement low in B_6, survived about 65 days from weaning with 100% incidence of severe dermatitis. With diet 787-B only three out of a group of eighty rats receiving little or no vitamin B_6 failed to develop dermatitis. Survival periods averaged about 100 days from weaning for the animals which developed dermatitis.

In the first experiments here reported we used diet 787-B. Using litter mates we tested the dried liver, and the alcohol-ether precipitate, the adsorbate and the filtrate alone or in combination, without added flavin. Figure 1 A shows the results. The experiment represented by curve IX was carried out somewhat later, but is included in this group for comparison. These rats received diet 793 supplemented with vitamin B, flavin, and the precipitate.

All rats which received the liver precipitate were completely protected from dermatitis. When the precipitate was fed alone there was little gain, but when supplemented with flavin or the adsorbate and filtrate obtained from the supernatant liquid, the rats gained 8 to 10 gm./ week. Animals receiving dried liver in an amount equivalent to 0.25 gm. showed slight lesions, but those on the higher level were in excellent condition. The remaining rats developed severe dermatitis.

We have prepared a flavin concentrate from fresh hog liver. The filtrate (filtrate W) remaining after the adsorption in this process is comparatively low in vitamin B_6, but it carries the

'filtrate factor.' When this filtrate is fed to rats receiving vitamin B and flavin, there is some growth, but the animals develop mild dermatitis. Addition of the liver precipitate in an amount equivalent to 1.0 gm. fresh liver induced rapid healing and a gain of 1 to 1.5 gm. per day (curve XI, fig. 1 B). These animals were receiving diet 793, with 200 γ daily of vitamin B concentrate.

As a final rather crucial test we gave the precipitate in a curative experiment. Out of forty rats which had received various materials in tests for their vitamin B_6 value, only seven had survived an 8-week experimental period. All had

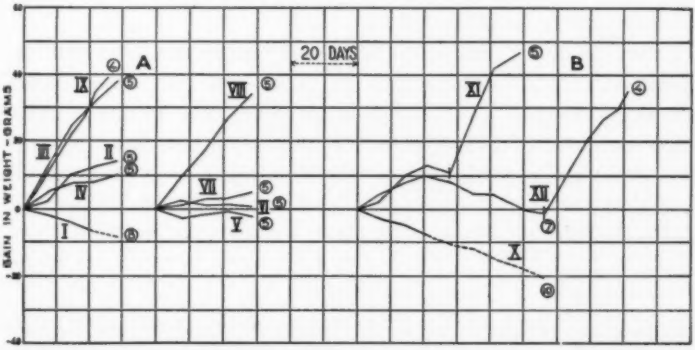

Fig. 1 A, curves representing average growth of rats which received diet 787-B plus 200 γ of vitamin B_1 with I, no additional supplement; II and III, dried liver equivalent to 0.25 and 0.5 gm. fresh material; IV, alcohol-ether precipitate equivalent to 1.0 gm. fresh liver; V, adsorbate from supernatant liquid equivalent to 1.0 gm. fresh liver; VI, filtrate resulting after adsorption equivalent to 1.0 gm. fresh liver; VII, the adsorbate and filtrate in combination equivalent to 1.0 gm. fresh liver each; VIII, adsorbate and filtrate supplemented by the precipitate equivalent to 1.0 gm. fresh liver each; IX, curve representing average growth of rats receiving diet 793 plus vitamin B_1, 5 γ crystalline lactoflavin, and the liver precipitate equivalent to 1.0 gm. fresh material.

B, curves representing average growth of rats which received diet 793 plus 200 γ of vitamin B_1 and X, no additional supplement; XI, filtrate W equivalent to 3.0 gm. fresh liver and 5 γ lactoflavin and at | the ether-alcohol liver precipitate equivalent to 1.0 gm. was added; XII, 5 γ lactoflavin and various materials under test (as described in text). At | the alcohol-ether liver precipitate was substituted for the test material. Dotted line indicates that one (or more) rats have died. The curve from this point represents growth of surviving animals. The numeral at the end of each curve indicates the number of rats of which results are included in the curve.

severe dermatitis, the ears were swollen and sore, there was a large raw area around the nose and mouth, the feet and forelegs were edematous and sore, and there was diarrhoea and a bloody urine. The rats had received vitamin B and flavin as well as the supplementary material in addition to diet 793. At the end of the experimental period the liver precipitate was substituted for the material which had been under test. Although these seven rats had received various supplements, their growth curves were similar and are averaged together. Of the group, three were too ill to respond and died within a few days after the precipitate was given, but the remaining four were rapidly cured and showed a gain of about 1.5 gm. per day (curve XII, fig. 1 B).

From these experiments we would conclude that the precipitate carries vitamin B_6. It is known that this vitamin can be adsorbed. It appears to be necessary to use a rather large amount of fuller's earth and a rather acid medium. The liver precipitate comes down as a mass of very fine particles, and it seems reasonable at least to assume adsorption of the vitamin on this material.

DISCUSSION

It is somewhat difficult to correlate these results with those of Elvehjem and co-workers. These authors state that rats which received their various basal diets supplemented by vitamin B only or by B, flavin, and 'B_2' showed no symptoms which might aid in determining the specific deficiencies. Since rats receiving our basal diets (particularly those which included sugar in place of starch) supplemented with B and flavin, uniformly developed dermatitis, specific of vitamin B_6 deficiency, it might seem that the diets used by these authors carried some vitamin B_6. In later work they included 12% white corn to provide vitamins B_4 and B_6, and in those diets dextrin was used in place of sugar. Here again there was no gain in weight, when supplemented by vitamin B, flavin and 'B_2,' and the animals were reported to have shown no symptoms other than lack of growth and general emaciation. We have tested the vitamin B_6 value of ground whole wheat,

but not corn. When rats received diet 793 supplemented by vitamin B and flavin, the addition of 1.0 gm. per day of whole wheat induced a gain averaging 0.6 gm. daily. It was not possible to obtain food consumption records with a sugar diet, but rats receiving a cornstarch diet consumed about 6 gm. daily when making this rate of gain. If a corresponding consumption of the sugar diet is assumed, we would have a level of 14% whole wheat in the diet. Since Elvehjem and coworkers included white corn at 12% and used dextrin as the carbohydrate, the results should be comparable. In our hands even a whole wheat extract, prepared according to the Bour-

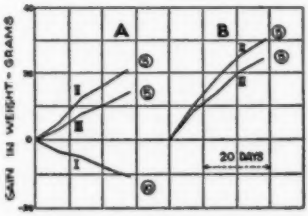

Fig. 2 A, curves representing average growth of rats which received diet 793 with 200 γ of vitamin B_1 daily and I, no additional supplement or B_1 and 5 γ of lactoflavin; II, 1.0 gm. daily of whole wheat plus 5 γ of lactoflavin; III, whole wheat extract equivalent to 4.0 gm. whole wheat daily and 5 γ of lactoflavin.

B, curves representing average growth of these rats when plotted as gain above that of litter mates receiving vitamin B_1 or B_1 and lactoflavin.

Dotted line indicates that one (or more) rats have died. The curve from this point represents growth of surviving animals. The numeral at the end of each curve indicates the number of rats of which results are included in the curve.

quin-Sherman ('31) method, induced a gain of about 0.5 gm. per day when fed in amounts equivalent to 4.0 gm. of whole wheat and supplemented by vitamin B and flavin (fig. 2).

Differences in the diet fed to stock rats in different laboratories will undoubtedly lead to different results in the experimental animals. However, table 1 gives results obtained by Copping ('36), by Birch, György and Harris ('35) and ourselves when white or yellow corn, whole wheat, or whole wheat extract are fed to rats receiving diets deficient in the B vitamins, and supplemented with crystalline or highly purified concentrate of vitamin B and with pure flavin. These are

TABLE 1

Comparisons of results from various laboratories from tests for the vitamin B_2 potency of whole wheat and corn

RESULTS REPORTED BY	CARBOHYDRATE IN BASAL DIET	SUPPLEMENT TO DIET	TEST MATERIAL	AVERAGE WEEKLY GAIN, GM.	RESULT
Copping	Maize sugar	Peters B_1 concentrate and 12 γ hepaflavin	Alcohol extract of maize or wheat 4.0 gm.	11.5	Perfect cure of florid dermatitis
Birch, György and Harris	Rice starch	Crystalline or highly purified B_1 and 10 γ lactoflavin	Maize, yellow		
			5.0 gm.	17.0	Perfect cure of florid dermatitis
			3.0 gm.	14.0	Perfect cure of florid dermatitis
			2.0 gm.	9.0	Perfect cure of florid dermatitis
			1.0 gm.	7.0	Perfect cure of florid dermatitis
			0.75 gm.	6.0	Perfect cure of florid dermatitis
			Maize, white		
			2.0 gm.	8.0	Perfect cure of florid dermatitis
			1.0 gm.	7.0	Perfect cure of florid dermatitis
			0.5 gm.	2.0	Perfect cure of florid dermatitis
			Whole wheat (ground)		
			3.0 gm.	12.0	Perfect cure of florid dermatitis
			2.0 gm.	12.0	Perfect cure of florid dermatitis
			1.5 gm.	8.0	Perfect cure of florid dermatitis
			1.0 gm.	4.0	Inconstant
Present authors	Sucrose	200 γ vitamin B_1 concentrate with 5 γ lactoflavin	1.0 gm.	4.2	No dermatitis
			Alcohol extract of whole wheat $\simeq$ 4.0	3.0	No dermatitis
Elvehjem and co-workers	Dextrin	B_1 concentrate, flavin and 'B_2'	12% white corn in diet	....	Protection from dermatitis. No gain in weight

compared with the results of Elvehjem and co-workers when 12% white corn is included in the diet, and vitamin B, flavin and 'B_2' are fed.

In the experiment reported by Copping, the rats were depleted up to 14 weeks, and had practically maintained their weight. Birch, György and Harris stated that about 7 weeks were required to induce dermatitis in animals. In our experiments, the rats developed symptoms in 3 to 4 weeks, and unless supplemented, died on an average of 9 to 10 weeks after weaning, with an average loss of 10 to 20 gm. in weight. The gain of rats receiving daily 1 gm. of whole wheat or whole wheat extract equivalent to 4 gm., above that of litter mate controls, receiving vitamin B or B and flavin, averaged 7.0 and 6.0 gm. per week, respectively, thus falling between the results from the other two laboratories. As seen in table 1 the results of Elvehjem and co-workers are very different.

From our results it appears that the cure of dermatitis in the rats resulting from feeding the liver precipitate was due to vitamin B_6 in this material. Whether the excellent rate of gain induced by the precipitate when fed with vitamin B and flavin or adsorbate (with or without the liver filtrate) was due to a new factor or to the combined action of vitamin B_6, flavin and the 'filtrate factor,' further work alone will show. In the experiments represented by curves IX and XII (fig. 1), no 'filtrate factor' was provided. However, in the first case the rats had been subjected to only a 24-day depletion period before the additional supplement was fed. In the second, the rats had been receiving various fractions from liver filtrate W prepared in an attempt to concentrate vitamin B_6. In both groups the body store of the 'filtrate factor' was undoubtedly sufficient to allow growth. This is confirmed by curve XI (fig. 1) where a similar rate of gain is obtained when filtrate W was fed with the precipitate and flavin. Also since we fed the crude precipitate without further purification, it seems probable that some 'filtrate factor' may have been present in the material.

From the liver we used and the procedure followed, it would seem that we obtained a fairly clear separation of flavin and

vitamin B_6. From the results of Elvehjem and co-workers it appears that some, at least, of the original liver flavin had been adsorbed by the precipitate, since rapid growth resulted from feeding the precipitate as the sole supplement to their white corn diet.

The lack of growth obtained by Elvehjem and co-workers when the white corn diet was supplemented with flavin and 'B_2' is difficult to understand, unless the flavin was not given at a sufficiently high level. In our work we used crystalline lactoflavin, as did also the English workers.

It is also difficult to understand why the rats receiving their various basal diets (without corn) should have died without showing symptoms of vitamin B_6 deficiency if the vitamin B concentrate was adequate. György ('35) found that too low a dose of vitamin B delayed the onset of vitamin B_6 symptoms. We tested each lot of vitamin B concentrate and fed it at the level which would induce a gain of at least 1 to 1.5 gm./day in rats receiving the Chase and Sherman ('31) vitamin B deficient diet.

The difference in results may be due to biological variations in the experimental animals. If such is the case, it will be difficult for one laboratory to confirm results obtained in another.

CONCLUSIONS

Experiments were carried out in an attempt to confirm the results of Elvehjem and co-workers regarding the new essential dietary factor found in mammalian liver. Our results appear to indicate that the alcohol-ether precipitation procedure carries down vitamin B_6, since the precipitate is highly effective in preventing and curing severe dermatitis.

We appear to obtain a separation of flavin and vitamin B_6 by this method, the flavin passing into the supernatant liquid.

Our results do not demonstrate a new factor essential in addition to vitamins B(B_1), flavin, B_4, B_6 and 'B_2' as postulated by these workers. However, until or unless results obtained in one laboratory can be duplicated in another, it is difficult to draw absolute conclusions.

ADDENDUM

A copy of this manuscript has been submitted to Professor Elvehjem. In a personal communication he states in part, "The results you have tabulated interest me very much because they merely add more evidence to our conclusion that liver contains a new essential dietary factor." His interpretation is as follows:

Our rations were developed with the idea of producing a vitamin B_6 deficiency. Our casein was lower in vitamin B_6 and higher in the alcohol precipitate factor than his. Due to this fact we produced more severe vitamin B_6 deficiency in a shorter time than most other laboratories.

Our results with air dried liver check those of Birch, György and Harris. These authors, however, found that it required an amount of liver extract equivalent to more than 5.0 gm. of fresh liver to equal 1 'rat day dose' of vitamin B_6. He emphasizes the fact that they (Elvehjem and co-workers) worked with liver extract. When this extract was fed at a level of 5% ($\simeq$ 6.0 gm. fresh liver daily) with a sucrose diet, excellent growth resulted. With a 2% level ($\simeq$ 2.4 gm.) the rats manifested symptoms of vitamin B_4 and B_6 deficiencies. For this reason they included 12% white corn. With liver extract $\simeq$ 2.4 gm., rats gained 100 gm. in 30 days whereas our results show a gain of only 40 gm. in 30 days when rats received 0.5 gm. fresh liver daily. (In experiments not included in this paper we obtained a gain of approximately 53 gm. in 30 days when 1.0 gm. equivalent of liver was fed, which is about half the gain Elvehjem and co-workers obtained with 2.4 gm.) Elvehjem states that obviously 0.5 gm. fresh liver does not supply enough alcohol precipitate factor for optimum growth, whereas liver extract $\simeq$ 2.4 gm. daily does supply enough.

With regard to the gain we obtained with whole wheat extract $\simeq$ 4.0 gm. whole wheat, he states that if our ration had not been low in the alcohol precipitate, the gain should have been 3 gm. per day. He states that, using 12% wheat, his results were similar to those obtained with corn. The higher gains obtained by the English workers he considers

were due to the fact that their rations or the grains were higher in this factor.

He concludes: "The fact that you found the alcohol-ether precipitate fraction from whole liver to be a good source of vitamin B_6 does not invalidate our work on a new factor found in liver extract. However, your work does make a definite addition to our knowledge of vitamin B_6."

LITERATURE CITED

BIRCH, T. W., P. GYÖRGY AND L. J. HARRIS 1935 The vitamin B complex. Differentiation of the antiblack tongue and the 'P-P' factor from lactoflavin and vitamin B_6 (so-called 'rat pellagra' factor). Parts I–VI. Biochem. J., vol. 29, p. 2830.

BOURQUIN, A. AND H. C. SHERMAN 1931 Quantitative determination of vitamin G(B_2). J. Am. Chem. Soc., vol. 53, p. 3501.

CHASE, E. F. AND H. C. SHERMAN 1931 A quantitative study of the determination of the antineuritic vitamin B. J. Am. Chem. Soc., vol. 53, p. 3506.

COPPING, A. M. 1936 The water soluble vitamins. V. Note on the two types of skin lesion occurring in vitamin B_2 deficiency in the rat in relation to deficiency of flavin and vitamin B_6, respectively. Biochem. J., vol. 30, p. 845.

ELVEHJEM, C. A. AND C. J. KOEHN, JR. 1935 The non-identity of vitamin B_2 and flavins. J. Biol. Chem., vol. 108, p. 709.

ELVEHJEM, C. A., C. J. KOEHN, JR. AND J. J. OLESON 1936 a An essential dietary factor found in yeast and liver extract distinct from vitamin B_1, B_2, B_4 and flavin. J. Biol. Chem., vol. 114, p. xxxi.

———— 1936 b A new essential dietary factor. J. Biol. Chem., vol. 115, p. 707.

GYÖRGY, P. 1935 Investigations on the vitamin B_2 complex. I–III. Biochem. J., vol. 29, p. 741.

HALLIDAY, N. AND H. M. EVANS 1937 Dietary production of the syndrome of deficiency in vitamin B_6. J. Nutrition, vol. 13, p. 657.

KOEHN JR., C. J. AND C. A. ELVEHJEM 1936 Studies on vitamin G(B_2) and its relation to canine black tongue. J. Nutrition, vol. 11, p. 67.

LEPKOVSKY, S. AND T. H. JUKES 1935 The vitamin G requirements of the chick. J. Biol. Chem., vol. 111, p. 119.

———— 1936 a The effect of some reagents on the 'filtrate factor' (a water-soluble vitamin belonging to the vitamin B complex and preventing a dietary dermatitis in chicks). J. Biol. Chem., vol. 114, p. 109.

———— 1936 b The distribution of the 'filtrate factor.' J. Biol. Chem., vol. 114, p. 117.

LEPKOVSKY, S., T. H. JUKES AND M. E. KRAUSE 1936 The multiple nature of the third factor of the vitamin B complex. J. Biol. Chem., vol. 115, p. 557.

SUPPLEE, G. C., G. E. FLANIGAN, Z. M. HANFORD AND S. ANSBACHER 1936 Lactoflavin. A possible contaminant of vitamin-free diets. J. Biol. Chem., vol. 113, p. 787.

VITAMIN B AND G VALUES OF PEAS AND LIMA BEANS UNDER VARIOUS CONDITIONS[1]

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TWO FIGURES

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Differences in the vitamin B and G values found in the literature for the same food are not surprising if one considers that in making the determinations several technics may have been employed—some doses being protective, others curative, while others are designed to give weight maintenance and still others growth. Furthermore, the total amount of any vitamin present in plant material may depend on such factors as variety, stage of development and conditions of growth. Thus Bell and Mendel ('22) found spring wheat two to three times as rich in the vitamin B complex as winter wheat, while Spohn (Sherman and Smith, '31) reported that two varieties of apple differed as much as 15% in the vitamin B complex, a difference due to a variation in vitamin G rather than in vitamin B (Cornell University Agr. Expt. Sta., '30).

An example of the influence of the stage of development is furnished by Hunt and Krause ('31), who found that early pasture produced milk with a higher content of both of these vitamins than did mature pasture. Eddy, Kohman and Carlsson ('26), comparing several types of canned peas as a source of the vitamin B complex, concluded that the more mature the pea the greater the vitamin content, but since the criterion of maturity was the relative size of the peas, this conclusion

[1] Based on a thesis presented by Esther H. Funnell Phipard in partial fulfillment of the requirements for the degree of doctor of philosophy.

would scarcely seem warranted, for some varieties are small at maturity while others are large.

It is not clear at present to what extent soil and climatic conditions affect the vitamin B and vitamin G content of plants. Rowlands and Wilkinson ('30) found that grass seed from land fertilized with manure was more active in promoting growth in young rats than seed from artificially enriched land. McCarrison and Viswanath ('26) found wheat to be 15% richer in the vitamin B complex when cattle manure had been used than when mineral fertilizer was employed. In contrast to this are the findings of Hunt ('27), who checked the content of the vitamin B complex of wheat grown on soils to which had been added various organic and inorganic fertilizers, and found little difference due to the treatment. There was, however, wide variability in the vitamin B content from year to year, which Hunt ascribed to differences in climatic conditions. Hanning ('36) has recently reported a difference of 130% between the vitamin B content of two samples of canned strained peas grown under similar conditions in 2 different years, but much less difference in the vitamin B values of two samples of carrots and green beans, while beets and spinach were practically the same in the two samples tested.

With regard to the influence of ordinary cooking processes, widely different results have been reported. Vitamin G is known (Smith and Hendrick, '26; Chick and Roscoe, '30; Keenan, Kline, Elvehjem and Hart, '35) to be relatively stable to the temperatures of ordinary cooking processes and therefore no appreciable loss of this factor due to heating alone would be anticipated. Vitamin B, on the other hand, is liable to considerable destruction by heat, the extent depending mainly upon temperature, duration of heating, and hydrogen-ion concentration of the solution. Roscoe ('31) found no loss in cooking with regard to either the vitamin B or vitamin G of carrots; in fact the cooking appeared to increase the availability. Munsell and Kifer ('32) reported a 50% loss in vitamin B in broccoli when cooked for 15

minutes. Dye and Hershey ('28) found a 45% depreciation when peas were blanched 5 minutes and processed 40 minutes compared to one of 25% when blanching was omitted but the processing continued for 40 minutes. Sherman ('32) reported that when fluid milk was heated for 6 hours at 100°C. about one-fourth of the vitamin B was destroyed. In a study by Sherman and Burton ('26) of the influence of hydrogen-ion activity, tomato juice heated for 1 hour at 100°C. was found to lose vitamin B with the increasing alkalinity of the solution.

Little is reported in the literature about the effects of drying and storage on the vitamin B and vitamin G content of peas, beans and other foods which are used in the dried form. Salmon ('26) found a loss of about 50% in the potency of the vitamin B complex of velvet beans stored for 3 years, but could find no such difference in soy beans during a similar period.

One of the newer methods of preservation used for a great variety of foods is quick freezing. By this process fruits and vegetables can be prepared, washed, graded, packed and quickly frozen all within 5 to 6 hours of the time of harvesting. Although a considerable number of studies have been made on the vitamin C values of frozen foods, no reports regarding the effect of this treatment on the vitamin B and vitamin G have as yet appeared.

Another interesting treatment of seeds used extensively by eastern peoples is sprouting. Several varieties of beans are used in this way. The rapid synthesis of vitamins A and C in the germinating seed has been clearly established by studies on a wide variety of materials. Less is known, however, concerning possible changes in the vitamin B and vitamin G content of young seedlings. Miller and Hair ('28) found a small but measurable amount of the vitamin B complex in etiolated mung bean sprouts which had been grown 4 days according to the custom of Orientals living in Hawaii. Since a liberal use of sprouted seeds in the diet will contribute very significant amounts of two important dietary essentials, vitamin A and vitamin C, it would be of interest to know what kind of

contribution of vitamin B and vitamin G such a food could make to the dietary.

The purpose of this study was to determine quantitatively the vitamin B and vitamin G content of two seeds important as foods, the pea (Pisum sativum) and the Lima bean (Phaseolus limenanus), in the green state, when raw, cooked and stored under freezing conditions; also when mature. The effect of sprouting the mature pea was also measured.

The peas used were two common varieties, Thomas Laxton and Alderman and the Lima beans were Henderson Bush, a dwarf variety known as the 'baby Lima' type. Fresh peas were obtained from the 1934 crop grown at Albion, New York, especially for commercial preservation. They were shipped at once after picking and were stored at 38°C. during the experimental period. Moisture determinations made weekly showed very little change, the average being 75.5%. Fresh Lima beans were grown by one of us from a sample of the seed used to produce the frozen ones. The first beans picked were slightly immature and showed a moisture content of 71.3% as compared to an average 65.8% for the later pickings. Since the feeding tests were begun on the younger beans, the amount fed of the more mature ones was adjusted to be equivalent in dry weight to that of the immature beans. The peas and beans as needed were shelled, ground in a food chopper and immediately weighed.

Frozen peas [2] were from the 1933 and 1934 crops and the frozen Lima beans were from the 1934 crop. They were kept in the original frozen packages at a temperature below freezing, a few being removed as needed and dried from 10 to 15 minutes on absorbent paper to remove the extraneous ice particles. Moisture determinations made after this treatment of the peas showed good agreement with those made on raw fresh ones, i.e., an average of 75.5% for both.

The mature pea and Lima bean seeds tested were samples of those used to grow the 1934 crops. Equal quantities of the

[2] The frozen peas, frozen Lima beans, fresh peas and pea and bean seed were kindly furnished by the Birdseye Frosted Foods Co.

two parieties of peas were mixed and then several lots of 50 and 100 seeds each were weighed in order to obtain an average figure for the weight of an individual seed. Both pea and Lima bean seeds were ground and mixed before the portions to be fed were weighed.

Sprouted peas were developed in a greenhouse [3] where the seeds (the same as those tested) were planted in flats of coarse washed sand and allowed to grow for 14 days, at which time the sprouts had attained a height of from 3 to 6 inches. The seedlings were then carefully removed from the flats, counted and washed free of sand and the seed remnants separated from the sprouts and roots. These two types of material, the residue of the seed and the new plant growth, were dried under an electric fan for 2 to 3 hours and then over night in a drying oven at 60°C.

Cooked peas were prepared as follows. A small quantity of the fresh (1934) peas was shelled, weighed and put into enough boiling water barely to cover them. They were cooked 15 minutes from the time they began to boil and were covered most of the time to prevent boiling dry. They were then weighed again and if necessary water was added to bring them to the original weight, so that 1 gm. of cooked peas was equivalent to 1 gm. of the raw material. The cooked peas were mashed with a fork to insure more uniform sampling, and were stored in a covered glass jar at a temperature of about 38°F.

Rats from mothers fed two-thirds whole wheat, one-third whole milk powder and sodium chloride equal to 2% of the weight of the wheat, plus lean beef three times per week, were weaned between the twenty-first and thirtieth day of age, when they weighed from 40 to 50 gm. They were housed individually in metal cages with raised screen bottoms of $\frac{1}{2}$ inch mesh. They had access at all times to distilled water and the basal diet, the consumption of which was recorded for each animal. Supplements were fed three times per week,

[3] Greenhouse facilities were made available through the courtesy of Archibald H. Funnell.

each portion being one-third of the amount required for 7 days. So far as possible equal numbers of males and females were used in each group of animals. Negative controls from nearly every litter were maintained on the basal diet.

VITAMIN B EXPERIMENTS

The basal diet lacking vitamin B was essentially that of Chase and Sherman ('31). It consisted of extracted casein, 18%; Osborne and Mendel salt mixture, 4%; cod liver oil, 2%; butter fat, 8%; cornstarch, 48% and autoclaved yeast, 20%. Two or three rats were kept in one cage until they were depleted, which occurred in an average of 16 days. Although a 4-week test feeding period would have been adequate, one of 5 weeks was selected since the 5-week figures were available. There was no significant difference in the results obtained at 4 and 5 weeks.

Frozen peas of the 1933 crop were fed at three levels, 0.5, 0.75 and 1.0 gm. daily, with resulting average gains for the 5-week period of 0.8, 4.7 and 9.2 gm. per week, respectively. When the fresh peas of the 1934 crop arrived, they were fed at a level of 0.5 gm., and gained on the average 7.2 gm. per week. When the 1934 peas were cooked as previously described and fed the same amount (0.5 gm.), the average gain was 5.3 gm. per week. This would indicate that a loss of 26% had been brought about by the process of cooking.

Since there was a difference between the vitamin B content of frozen peas of the 1933 crop and fresh peas grown in 1934, the latter crop was tested frozen as soon as the 1934 packing became available. With 0.5 gm. of these fed daily the animals showed an average gain of 7.6 gm. per week for the period. Therefore there was no loss of vitamin B due to the freezing process.

The mature pea seeds were fed in portions equivalent on a dry weight basis to the fresh peas fed, 0.13 gm. of ripe seed being equivalent to 0.5 gm. of fresh peas. The twelve rats so fed made an average gain of 4.2 gm. per week. The difference in average gain of 3 gm. per week indicates that the

vitamin B content of the dry mature pea seed was much lower than that of the fresh peas. The results are shown in table 1 and figure 1.

The fresh peas proved to be a rich source of vitamin B, containing approximately 3 Sherman-Chase units per gram.

TABLE 1

A summary of the growth response of animals used to determine the vitamin B content of peas and Lima beans

MATERIAL TESTED	AMOUNT FED DAILY	DRY WEIGHT EQUIVALENT	NUMBER OF RATS	AVERAGE GAIN IN 5 WEEKS	AVERAGE GAIN PER WEEK
	gm.	*gm.*		*gm.*	*gm.*
Frozen peas (1933)	0.50	0.12	7	3.8	0.8
	0.75	0.19	10	23.5	4.7
	1.00	0.25	4	45.8	9.2
Frozen peas (1934)	0.50	0.12	10	38.1	7.6
Fresh peas (1934)	0.50	0.12	10	35.9	7.2
Cooked peas (1934)	0.50	0.12	7	26.3	5.3
Pea seed (1933)	0.13	0.12	12	21.1	4.2
Sprouts and roots of one pea seed	0.09	....	8	22.9[1]	5.7
Seed residue of one pea seed	0.05	....	8	— 7.1[1]	— 1.8
Fresh Lima beans (1934)	1.00	0.29	7	35.2	7.0
	0.70	0.20	10	19.5	3.9
Frozen Lima beans	1.00	0.29	3	— 6.3[1]	— 1.6
Lima bean seed (1933)	0.31	0.28	9	16.8	3.4

[1] Four weeks.

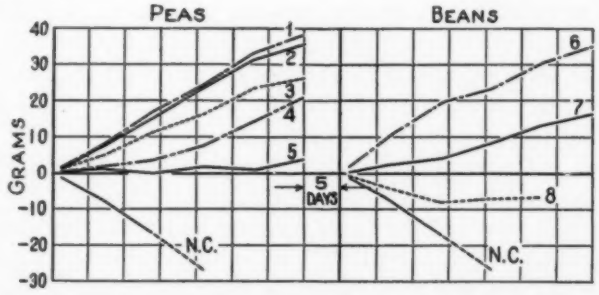

Fig. 1 Growth of animals receiving a vitamin B deficient diet plus daily supplements of the following: 1, 0.5 gm. frozen peas (1934); 2, 0.5 gm. fresh peas (1934); 3, 0.5 gm. fresh peas cooked; 4, 0.13 gm. pea seed equivalent to 0.5 gm. fresh peas; 5, 0.5 gm. frozen peas (1933); 6, 1.0 gm. fresh Lima beans (1934); 7, 0.31 gm. Lima bean seed equivalent to 1.0 gm. fresh Lima beans; 8, 1.0 gm. frozen Lima beans (1934); N.C., negative controls.

This is a higher vitamin B content than most vegetables which have been investigated. The fresh peas were found to be twice as rich in vitamin B as the fresh Lima beans. There was very good agreement between the vitamin B content of fresh and frozen peas from the same crop but not with the peas from different crops. The lesser amount of vitamin B in the frozen peas of the 1933 as compared with the 1934 crop cannot be attributed to prolonged storage since when tested they had been stored only 2 to 4 months.

In the studies of the effect of sprouting, in which ripened pea seeds were used as the basis of comparison, a daily dosage of 0.13 gm. of ripened pea seed was chosen as it represented approximately one-half of a fresh seed. An attempt was

TABLE 2

A comparison of the vitamin B content of sprouted and unsprouted pea seeds

MATERIAL	AMOUNT FED DAILY	NUMBER OF SEEDS REPRESENTED	NUMBER OF RATS	GAIN PER WEEK IN 4 WEEKS
	gm.			*gm.*
Pea seed	0.13	0.5	12	4.2
Sprouts and roots	0.09	1.0	8	5.7
Seed residue	0.05	1.0	8	— 1.8

made to feed the dried sprouts and roots together in amounts corresponding to one-half of a seed but the doses were too small to furnish measurable amounts of vitamin B, and therefore at the end of 1 week of feeding the quantities were doubled and the tests continued for 4 weeks. The seed residues were also fed in amounts corresponding to one seed. Thus it was possible to compare the vitamin B values of the new plant substance (sprouts and roots) with the unsprouted pea seed, and also with the remnants of the germinated seed. The eight rats which received the dried sprouts and roots equivalent to approximately one seed (0.09 gm. per day), made an average gain of 5.7 gm. per week, whereas their litter mates receiving the corresponding residue, lost weight, indicating much destruction of vitamin B during the 14-day period of sprouting the seed. The results are shown in table 2.

The Lima beans were tested under three conditions, fresh, frozen and mature. When the fresh beans were fed in amounts of 0.7 and 1.0 gm. daily the average gains per week were 3.9 and 7.0 gm., respectively. Three rats each fed 1 gm. daily of the frozen Lima beans of the 1934 crop lost 6.3 gm. in 4 weeks. The mature seeds, some of which were used to grow the fresh beans, were fed in amounts equivalent on a dry weight basis to the daily dosage of fresh beans, with an average gain of 3.4 gm. per week for the period.

Since the fresh and frozen Lima beans were from two different crops, grown in two different localities, no comparison is possible with regard to the influence of freezing, but judging from the studies on the peas, the low vitamin B content of the frozen beans tested is in all probability due to climatic factors influencing growth and not to the freezing process.

The mature dried beans were less rich in vitamin B than the fresh seeds. It is not possible, however, to tell whether this is due entirely to maturity or partly to drying and storage, since the seed itself was grown in another locality and in the previous season. The experience with peas would indicate that maturity was the primary cause.

VITAMIN G EXPERIMENTS

The method used for the vitamin G studies was that of Bourquin and Sherman ('31) with an experimental period of 4 weeks. At the end of the first week of the depletion period, the rats were harnessed according to the anticoprophagy technic of Page ('32). Frozen peas of the 1933 crop were fed in amounts of 0.75, 1.0 and 1.5 gm. daily with resulting average gains of 1.7, 3.2 and 5.2 gm. per week, respectively. When the fresh peas of the 1934 crop became available, one group of ten rats was fed 1 gm. daily of the raw peas with an average gain of 4.4 gm. per week for the period, and another group of seven rats consuming the same peas cooked made an average gain of 4.2 gm. per week. A comparison of these results in figure 2 shows that there was

little difference in the vitamin G content of fresh, frozen and cooked peas of the same crop. There was, however, the same kind of difference between the vitamin G content of the frozen peas of the 1933 and 1934 crops as was shown in the vitamin B studies, the 1934 crop being richer in each case.

The mature pea seed (1933 crop) fed to a group of thirteen rats in doses of 0.27 gm. daily, equivalent to 1 gm. of fresh peas on a dry weight basis or one seed, resulted in an average gain of 3.4 gm. per week, as compared with 4.4 gm. on fresh peas.

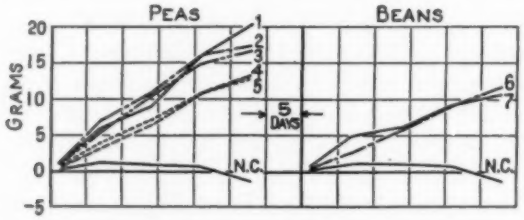

Fig. 2 Growth of animals receiving a vitamin G deficient diet plus daily supplements of the following: 1, 1.0 gm. frozen peas (1934); 2, 1.0 gm. fresh peas (1934); 3, 1.0 gm. fresh peas cooked; 4, 0.27 gm. pea seed equivalent to 1.0 gm. fresh peas; 5, 1.0 gm. frozen peas (1933); 6, 1.0 gm. fresh Lima beans; 7, 0.31 gm. Lima bean seed equivalent to 1.0 gm. fresh Lima beans; N.C., negative controls.

The sprouted pea seedlings were used in the way previously described for the vitamin B studies. For comparison mature pea seed was fed to three groups of rats on the basis of three-fourths, one, and one and one-half seeds daily producing average gains of 2.8, 3.5 and 5.1 gm. per week, respectively. To measure the amount of vitamin G in the sprouts and roots combined, the equivalent of one and one-half seeds a day was chosen. This amount of sprouts and roots produced an average gain of 4.9 gm. per week. The seed residue from one and one-half sprouted peas was also tested with the resulting gain of 2.9 gm. per week. The newly formed plant material proved to be as good a source of vitamin G as the original seed and the seed remnant still contained an appreciable amount of the vitamin.

The fresh Lima beans were fed in a dosage of 1 gm. daily and produced an average gain in ten rats of 3.9 gm. per week. When mature Lima bean seeds were fed on an equivalent weight basis, with 0.31 gm. of the dry seed as the daily dosage, there was an average gain of 2.7 gm. per week. Therefore there was practically no difference in potency between the fresh green beans and the mature seed. Table 3 summarizes the data for these tests and figure 2 shows the growth curves.

TABLE 3

Growth response of animals used to determine the vitamin G content of peas and Lima beans

MATERIAL TESTED	AMOUNT FED DAILY	DRY WEIGHT EQUIVALENT	NUMBER OF RATS	AVERAGE GAIN IN 4 WEEKS	AVERAGE GAIN PER WEEK
	gm.	*gm.*		*gm.*	*gm.*
Frozen peas (1933)	0.75	0.18	5	6.8	1.7
	1.00	0.25	5	12.8	3.2
	1.50	0.37	2	21.0	5.2
Frozen peas (1934)	1.00	0.25	7	20.4	5.1
Fresh peas (1934)	1.00	0.25	10	17.5	4.4
Cooked peas (1934)	1.00	0.25	7	16.9	4.2
Pea seed (1933)	0.27	0.25	13	13.4	3.4
Pea seed (1.5 seeds daily)	0.43	....	3	20.3	5.1
Sprouts and roots of 1.5 pea seed	0.14	....	10	19.5	4.9
Seed residue of 1.5 pea seed	0.11	....	8	11.8	2.9
Fresh Lima beans	1.00	0.29	10	11.8	3.0
Lima bean seed (1933)	0.31	....	8	10.9	2.7

Both fresh peas and fresh Lima beans proved to be fairly good sources of vitamin G, containing approximately 1 Sherman-Bourquin unit per gram. They were not as rich in vitamin G, however, as in vitamin B, thus furnishing additional evidence that seeds generally are relatively richer in vitamin B than in vitamin G. Fully ripened pea and Lima bean seeds were found to be about as rich in vitamin G as the fresh green ones, but further investigation of this point is desirable since the samples used were grown in different localities and under different conditions.

When peas were sprouted and allowed to grow for 2 weeks, there was a larger amount of vitamin G in the seedling, 3 to 6 inches high, than in the original seed.

SUMMARY

The vitamin B and G content of fresh peas has been determined for raw, cooked and frozen material and for mature seeds dry and sprouted; also for fresh Lima beans, both raw and frozen and for the mature seeds. Fresh raw peas were found to be a rich source of vitamin B, containing approximately 3 Sherman-Chase units per gram. Peas of the same season showed no loss due to freezing but a 26% loss in cooking 15 minutes. Peas of two seasons differed by almost 100%. Fresh Lima beans were only half as potent in vitamin B as fresh peas. Maturity in both peas and Lima beans resulted in loss of approximately half of the vitamin B.

Seedling peas grown on pure sand for 14 days showed loss of over half of the vitamin B but synthesis of vitamin G, the newly formed plant material being as good a source as the original seed, and the seed residue still containing a considerable amount of the vitamin.

Differences between fresh and frozen Lima beans of the same variety and season but grown in different regions show that locality must be taken into account in vitamin studies. Seasonal differences in vitamin G appeared in the frozen peas of the two crops studied just as in case of vitamin B.

The vitamin G content of both fresh raw peas and Lima beans was the same, 1 Sherman-Bourquin unit per gram. There was no loss of vitamin G in either in cooking or freezing.

In mature seeds of both kinds there was little loss of vitamin G as compared with the fresh seed.

LITERATURE CITED

BELL, M. AND L. B. MENDEL 1922 The distribution of vitamin B in the wheat kernel. Am. J. Physiol., vol. 62, p. 145.

BOURQUIN, A. AND H. C. SHERMAN 1931 Quantitative determination of vitamin G(B_2). J. Am. Chem. Soc., vol. 53, p. 3501.

CHASE, E. F. AND H. C. SHERMAN 1931 Quantitative study of the determination of the antirachitic vitamin B. J. Am. Chem. Soc., vol. 53, p. 3506.

CHICK, H. AND M. H. ROSCOE 1930 Heat-stability of the (anti-dermatitis, 'anti-pellagra') water-soluble vitamin B_2. Biochem. J., vol. 24, p. 105.

CORNELL UNIVERSITY AGRICULTURAL EXPERIMENT STATION 1930 Vitamin investigations. Ann. Rept., p. 105.

DYE, M. AND R. HERSHEY 1928 The effect of canning on the vitamin A and B content of peas. Preliminary report (abstract). J. Home Econ., vol. 20, p. 761.

EDDY, W. H., E. F. KOHMAN AND V. CARLSSON 1926 Vitamins in canned foods. IV. Green peas. Ind. Eng. Chem., vol. 18, p. 85.

HANNING, F. 1936 Further studies of the content of vitamins A and B in canned strained vegetables. J. Am. Dietet. Assoc., vol. 12, p. 231.

HUNT, C. H. 1927 Vitamin B in wheat as influenced by fertilizers. Ohio Agr. Exp. Sta. Bull., vol. 415.

HUNT, C. H. AND W. E. KRAUSS 1931 Influence of the ration of the cow upon the vitamin B and vitamin G content of milk. J. Biol. Chem., vol. 92, p. 631.

KEENAN, J. A., O. L. KLINE, C. A. ELVEHJEM AND E. B. HART 1935 The stability of vitamins B(B_1), G(B_2) and B_6. J. Nutrition, vol. 9, p. 63.

MCCARRISON, R. AND B. VISWANATH 1926 The effect of manurial conditions on the nutritive and vitamin values of millet and wheat. Ind. J. Med. Res., vol. 14, p. 351.

MILLER, C. AND D. HAIR 1928 The vitamin content of mung bean sprouts. J. Home Econ., vol. 20, p. 263.

MUNSELL, H. E. AND H. B. KIFER 1932 The vitamin B and G content of raw and cooked broccoli. J. Home Econ., vol. 24, p. 823.

PAGE, J. W. 1932 An improvement in experimental method for the investigation of vitamin G. Proc. Soc. Exp. Biol. and Med., vol. 30, p. 87.

ROSCOE, M. H. 1931 The distribution of the vitamin B complex. II. Root vegetables. Biochem. J., vol. 25, p. 1205.

ROWLANDS M. J. AND B. WILKINSON 1930 The vitamin B content of grass seeds in relationship to manures. Biochem. J., vol. 24, p. 199.

SALMON, W. D. 1926 Factors affecting the vitamin B content of plant products. Ala. Exp. Sta. Rept., p. 15.

SHERMAN, H. C. 1932 Chemistry of food and nutrition, 4th ed. The Macmillan Co., p. 391.

SHERMAN, H. C. AND G. W. BURTON 1926 Effect of hydrogen-ion concentration upon the rate of destruction of vitamin B. J. Biol. Chem., vol. 70, p. 639.

SHERMAN, H. C. AND S. L. SMITH 1931 The vitamins, 2nd ed. The Chemical Catalogue Co., p. 94.

SMITH, M. I. AND E. G. HENDRICK 1926 Some nutrition experiments with brewers' yeast with especial reference to its value in supplementing certain deficiencies in experimental rations. U. S. Pub. Health Repts., vol. 41, p. 201.

THE EFFECT OF THE ACID-BASE CONTENT OF THE DIET UPON THE PRODUCTION AND CURE OF RICKETS WITH SPECIAL REFERENCE TO CITRATES

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ONE FIGURE

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The question of the acid-base factor in the etiology and pathogenesis of rickets is one that has long excited interest. The experimental results have, however, been conflicting and the controversial discussions have failed to clarify the situation. Summaries of the earlier work have been given by Shelling ('25), Hess ('29), György ('29) and Goldblatt ('31) and in our earlier papers (Shohl et al., '28, Shohl et al., '32).

That rickets is associated with an acid metabolism and tetany with an alkaline, has been strongly advocated by Freudenberg and György ('22). The role of acidosis in the pathogenesis of rickets has recently been reviewed by Morris, Ford and Graham ('36) who could find no evidence that acidosis was "either a causal or associated factor in infantile rickets." The acid-base equilibrium of the blood shows little or no variation from the normal in either clinical rickets or infantile tetany, or, except in special cases, in experimental rickets (Shohl et al., '31). Therefore, efforts to show changes in metabolism have been directed toward studies of the excretion of acid and ammonia in the urine.

With regard to the effect of ingestion of acid our previous experiments have tended to confirm György's thesis, with

the reservation that, in the absence of vitamin D, the acid-base factor is secondary to alteration of the calcium-phosphorus intake. Obviously the acid-base content of the diet cannot be important clinically because the diet of infants, milk, is alkaline. Further, the acid-base effects could be shown experimentally in border line diets only. Third, it was evident that the acid-base effects were not direct but were complicated by the separate effects upon absorption and metabolism.

This last conclusion was based upon the following observations and reasoning. In rachitic tetany acid tends toward amelioration and alkali intensifies the condition. However, when acid, neutral and alkaline phosphates were given to rachitic rats, not the alkaline diets but the neutral ones produced the most severe tetany. From this we concluded that the total effect must be the resultant of two processes: 1) acid increased the absorption of Ca and P; alkali prevented it, 2) in metabolism after absorption, acid removed Ca and P from the body and alkali favored their deposition in the bones.

Hamilton and Schwartz ('33) have extended this thesis by a clever method of separating the two factors. They added $(NH_4)_2CO_3$ and NH_4Cl to 'normal' diets which were thus rendered alkaline in the intestinal tract and acid in metabolism. This alteration sufficed to make the diets rickets producing. It follows that a reversal of the factors should cause an opposite physiological effect. Thus, with diets which were acid in reaction but had an alkaline ash, namely with the addition of tartaric acid and sodium tartrate to diets which otherwise caused rickets, no rickets resulted. Their results are shortly to be published in detail.

Because the tartrates are too toxic to be used clinically it seemed desirable to extend this study to other organic acids. Further, we have recently shown (Brown et al., '32; Querido, '35; Shohl, '36) that when both the absolute amounts and the ratios of Ca and P are considered, rickets can be produced with many types of diets other than the classical high calcium-low phosphorus diets. Therefore, it was necessary to investigate whether the same acid-base effects would be obtained

in conjunction with different types of diet. It should then be possible to determine whether the acid-base alternations alone can be responsible for the etiology and also the cure of rickets in rats.

PLAN OF EXPERIMENT

Young albino rats were reared in our laboratory from Wistar Institute stock. The mothers were fed Sherman diet B (66% entire wheat flour, 33% dried whole milk, 1.3% NaCl) throughout pregnancy and lactation. The young were weaned to the same diet at 21 days. At 28 days of age, when weighing 45 to 55 gm., they were changed to the experimental diets and kept in groups of three in wide meshed cages without any bedding. They were fed these diets and distilled water. They were weighed weekly. After 21 days they were x-rayed, bled under light ether anesthesia, and autopsied. The bones of one hind leg were preserved for histological examination and those of the other carefully dissected, weighed, and the femur used for ash determination of the alcohol-ether extracted dry bones. The Ca and P of the serum were determined by the methods of Fiske and Logan ('31), and Fiske and Subbarow ('25), respectively. The individual x-rays were read and at the end of the experiment all the roentgenograms were compared and the original diagnoses checked and verified. The histological examinations and diagnoses therefrom were made without knowledge of the diets employed or the x-ray findings, through the kind cooperation of Dr. S. B. Wolbach.

As in our previous experiments, the two methods of diagnosis were in such close agreement that the results will not be differentiated in the following report.

The experimental diets consisted of 80% ground corn, 20% gluten flour and 1% NaCl, with additions of $CaCO_3$ and KH_2PO_4 to give the desired levels and ratios of Ca and P, as has been fully described in our previous paper (Shohl, '36). Diets containing any of these ratios were rickets producing provided the absolute amounts of Ca and P were low,

but were non-rachitogenic if the levels were high. The border line between rachitogenic and non-rachitogenic diets is shown in figure 1. The salt additions were made equivalent by computing them in terms of cubic centimeters of normal solutions per 100 gm. of diet, and were added as dry salts to the dry diet, and fed ad libitum. In a few cases the food intakes of individual rats were measured. These did not vary from those usually observed on similar diets so the procedure was discontinued. 1) Rickets production. $(NH_4)_2CO_3$ and NH_4Cl

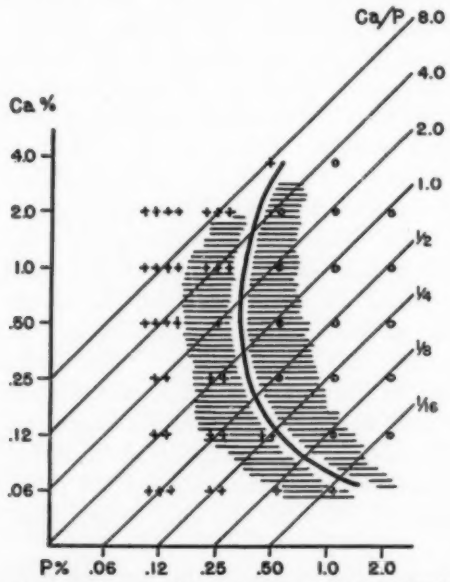

Fig. 1 Effect of alterations in the diet upon rickets production and rickets prevention. The abscissa and ordinate are laid off logarithmically to represent the diet percentages of P and Ca, respectively, and each point chosen is double that of the previous point. The curve represents the dividing line between rachitogenic diets on the left and non-rachitogenic diets on the right as described in our previous paper (Shohl, '36). The shaded area to the left of the line shows the extent of the field of successful rickets prevention with the use of citric acid-sodium citrate mixtures, or intensification of the degree of rickets by $(NH_4)_2CO_3$–NH_4Cl mixtures. The shaded area to the right of the line represents that in which non-rachitogenic diets were converted to rachitogenic diets by the use of $(NH_4)_2CO_3$–NH_4Cl mixtures, as described in the text.

were added to the diets. To test the effect of the component salts they were added separately and in combination in varying amounts to diet L (Ca = 0.5%, P = 0.5%) which is non-rachitogenic. The most effective combination was added to the diets either side of the border line in each of the eight ratio zones as shown in figure 1. 2) Rickets prevention. A citric-acid-sodium citrate mixture was added to the rachitogenic diet, St (Ca = 1.0%, P = 0.25%), separately and in combination. The mixture which was most effective was added to each of the border line rachitogenic diets. When the effects of these diets had been determined other organic acids—lactic, acetic, succinic, malic, malonic and tartaric were tested, using only one basal diet, that which most closely approached the classical diet no. 2965 of Steenbock and Black (diet St).

RESULTS

1. Rickets production. $(NH_4)_2CO_3$ and NH_4Cl were added separately and in combination to diet L as shown in table 1. In making the diet rachitogenic it is evident that the combination of the salts was more effective than either alone. Of the two the NH_4Cl is the more important for the $(NH_4)_2CO_3$ alone had no effect, but the acid-producing salt alone, if given in sufficient amount, made the diet rachitogenic.

A combination of 600 cc. 0.1 N $(NH_4)_2CO_3$ and 600 cc. 0.1 N NH_4Cl was added to rachitogenic and non-rachitogenic border line diets indicated in table 2. Most of the data represent six animals on each diet, run in groups of three so as to afford independent checks. Where the diet originally was rickets producing, the acid intensified the effect in every case, as evidenced by x-ray and histological examination. Except in the case of diet Y, the bone ash percentages were the same or lower than those of the rats fed the basal diets. Where the diet was one which produced normal bones at or near the border line (i.e., containing double the amounts of Ca and P per given ratio necessary to produce rickets), in every case the addition of $(NH_4)_2CO_3 + NH_4Cl$ made the diet rachitogenic. Where the diet was two stages from the border line

(i.e., containing four times the amounts of Ca and P), as in diets Q and O, the x-rays did not show rickets. However, the bones were thinner and the ash lower. They were considered border line cases. In the case of diet Q, with the addition of three times as much acid a definitely osteoporotic bone was produced and the per cent of ash was further lowered without producing definite rickets.

The growth of all the experimental animals, in accordance with our previous experience, was less than normal and varied from no weight increase to 12 gm. for the 21 days. It was

TABLE 1

Effect of additions of $(NH_4)_2CO_3$ and NH_4Cl to a non-rachitogenic diet [1]

ADDITIONS TO DIET L [1]		BLOOD SERUM		RICKETS [2]
NH_4Cl	$(NH_4)_2CO_3$	Ca	P	
cc. 0.1N per 100 gm. of diet	*cc. 0.1N per 100 gm. of diet*	*mg.%*	*mg.%*	
0	0	9.0	6.0	—
600	600	9.7	7.0	++
0	1200	9.7	7.6	—
600	0	6.6	6.0	+
1200	0	5.0	8.0	++

[1] Diet L which was used is described in text and in table 2.

[2] Histologic and x-ray diagnoses. The number of + signs indicates the severity of rickets; — indicates the absence of rickets.

impossible to correlate the growth with the presence or absence of rickets.

From the data given in table 1 it is obvious that blood serum values give no clue as to the presence or absence of rickets. However, when the acid-producing salt was the sole addition the calcium was reduced and this effect was intensified when the amount of acid was increased. Morgan ('34) observed low calcium with acid diets.

The conclusion follows: acid-producing salts intensify rickets and have a definite but limited effect on the production of rickets.

TABLE 2

Effect of $(NH_4)_2CO_3$–NH_4Cl and citric acid-sodium citrate mixtures on the production of rickets and the amount of bone ash

	COMPOSITION OF BASAL DIET				RICKETS [1]			BONE ASH [2]		
						Basal diet + additions [4]			Basal diet + additions [4]	
	Ca/P	Ca	P	Acid-base [3]	Basal diet	$(NH_4)_2CO_3$ + NH_4Cl	Citric acid + sodium citrate	Basal diet	$(NH_4)_2CO_3$ + NH_4Cl	Citric acid + sodium citrate
		%	%	*cc. 0.1N*				%	%	%
Y	8/1	4.00	0.5	— 1865	±	+	—	46	51	50
St	4/1	1.00	0.25	— 430	+++	++++	—	39	39	49
W	4/1	2.00	0.50	— 865	—	+		50	47	
S	2/1	0.50	0.25	— 130	+	+	—	46	47	42
X	2/1	1.00	0.50	— 365	—	+		57	46	
K	1/1	0.25	0.25	— 30	±	+	—	46	41	37
L	1/1	0.50	0.50	— 65	—	+	—	48	42	
Q	1/1	1.00	1.00	— 195	—	—		50	46	
F	1/2	0.12	0.25	+ 40	+	++	—	41	36	42
G	1/2	0.25	0.50	+ 45	—	++		47	42	
O	1/2	0.50	1.00	+ 85	—	—		46	43	
B	1/4	0.12	0.50	+ 105	+	++	—	36	34	42
I	1/4	0.25	1.00	+ 195	—	+		41	41	
C	1/8	0.06	0.50	+ 135	+		—	37		36
D	1/8	0.12	1.00	+ 255	+	++	—	35	34	37
E	1/16	0.06	1.00	+ 285	+	++	—	32	33	38

[1] Histologic and x-ray diagnoses. The number of + signs indicates the severity of rickets; — indicates the absence of rickets.

[2] Determinations made on dried, fat-free femurs. [3] Calculated per 100 gm. of diet. + = acid; — = base.

[4] Additions were: 600 cc. 0.1N $(NH_4)_2CO_3$ + 600 cc. 0.1N NH_4Cl per 100 gm. of diet, or 900 cc. 0.1N citric acid + 600 cc. 0.1N sodium citrate per 100 gm. of diet.

2. Rickets prevention. To analyze the action of citrates, variable amounts of sodium citrate and citric acid were added to diet St (Ca = 1.0%, P = 0.25%) separately and in combination (table 3). The remarkable effect of citrates in the prevention of rickets was not due to the citrate ion alone, for neither citric acid nor sodium citrate alone gave protection, either in the amounts used in combination with the

TABLE 3

Effect of additions of citric acid and sodium citrate to a rachitogenic diet [1]

ADDITIONS TO DIET ST [1]				RICKETS [2]
Calcium citrate [3]	Citric acid	Sodium citrate	Total	
	cc. 0.1N per 100 gm. of diet			
	0	0	0	+++
	300	300	600	+++
	0	600	600	++
	500	400	900	++±
	300	600	900	++
	900	0	900	++±
	1500	0	1500	++
	0	1500	1500	++
	900	600	1500	—
500	0	0	500	++±
500	0	400	900	++±
500	400	0	900	++
500	600	400	1500	+
500	400	600	1500	—

[1] Diet St which was used is described in the text and in table 2.

[2] Histologic and x-ray diagnoses. The number of + signs indicates the severity of rickets; — indicates the absence of rickets.

[3] Calcium citrate, where used, replaced an equivalent amount of calcium carbonate.

other, or when the total amount of citrate was given in either form.

The citrate combination which had been found most effective, 900 cc. 0.1 N citric acid and 600 cc. 0.1 N sodium citrate, was added to each of the rachitogenic diets close to the border line. Whether high alcium-low phosphorus, low calcium-high phosphorus, or low calcium-low phosphorus, the

diets which had previously produced rickets now caused no rachitic lesions, as evidenced by x-ray and histologic studies (table 2). The bone ash showed an increase in six cases and a diminution in two cases. No effort was made to explore the extent of the field of this salt effect, but the diets studied included the classical rickets producing diet. To attain lower phosphorus diets than those used it would have been necessary to diminish the P more than could be done with the use of corn, and such alterations of the diet would involve P starvation and other problems lying outside the field of this investigation.

The blood serum Ca and P showed no alteration from that of classical low P rickets when the citrate mixtures did not prevent rickets. With intermediate and effective amounts the P tended to rise irregularly and the Ca to be depressed.

The $CaCO_3$ present in diet St causes an excess of 430 cc. 0.1 N alkali per 100 gm. of diet. It was desired to test whether the neutralization of the carbonate outside the body was the same as that inside the body. Therefore, a diet was made with an equivalent amount of calcium citrate instead of the carbonate. Various additions of citric acid and sodium citrate were made to this diet as shown in table 3. In no case was the diet effective in preventing rickets until the original values (900 cc. citric acid and 600 cc. sodium citrate) were reached. Neither the acid nor the alkali alone was sufficient to bring about the prevention of rickets; both citric acid and sodium citrate were necessary. One must conclude that the factors of acid in absorption and alkali in metabolism cooperate in the total effect.

As a further test the curative action of the citrates was investigated. Rickets was produced by feeding animals diet St for 21 days. They were then fed, in addition, the citric acid-sodium citrate mixture shown above to be effective in the prevention of rickets. As early as 7 days following this addition to the diet, healing was evidenced by x-ray, serum analysis and ash determination.

If the results obtained with citrates were due solely to the acid-base effect on metabolism, it follows that similar effects should be produced with other organic acids and their salts. Accordingly a test was made with diet St using equivalent amounts of tartaric acid and sodium tartrate. The diet was rendered non-rachitogenic by these additions, as shown in table 4. This confirms the preliminary report of Hamilton, referred to above. However, when other acids, acetic, lactic,

TABLE 4

Effect of additions of various organic acids and their salts to a rachitogenic diet [1]

ADDITIONS TO DIET ST [1]	RICKETS [2]	BONE ASH [3]
cc. 0.1N per 100 gm. of diet		%
Diet St	+++	34
900 citric acid + 600 sodium citrate	—	49
900 tartaric acid + 600 sodium tartrate	—	38
Lactate [4]	+++	31
900 lactic acid + 600 sodium lactate	+++	35
Lactate [4] + 900 lactic acid + 600 sodium lactate	++	40
900 acetic acid + 600 sodium acetate	+++	36
900 malic acid + 600 sodium malate	+++	
900 malonic acid + 600 sodium malonate	+++	
900 succinic acid + 600 sodium succinate	+++	

[1] Diet St which was used is described in the text and in table 2.

[2] Histologic and x-ray diagnoses. The number of + signs indicates the severity of rickets; — indicates the absence of rickets.

[3] Determinations made on the dried, fat-free femurs.

[4] In these cases the $CaCO_3$ of diet St was replaced by an equivalent amount of calcium lactate.

malic, malonic and succinic acids, and their sodium salts were used, the diet still produced rickets, as evidenced by both x-ray and histologic examination, and the blood serum showed the findings characteristic of low phosphorus rickets. Although the acid-base content of the diet is important, for, only in the presence of the acid and its salt are the citrates effective in rickets prevention, the results obtained with citrates and tartrates must be due to their specific properties and not to their acid-base effect alone.

DISCUSSION

Schloss ('17) objected to the acid theory of rickets, on the basis that acid ingestion leads to osteoporosis and not to rickets, and second that alkali administration does not heal rickets. For experimental rickets these objections are no longer valid.

From the data presented it is clear that the acid-base content of the diet is important in the etiology of experimental rickets in rats. In our previous experiments (Shohl et al., '32) the diets chosen for study were so near the border line that they should not strictly be classed as either rachitogenic or non-rachitogenic. With neutral diets a mild healing type of the condition was present. Under these conditions acid diets intensified rickets and alkaline diets prevented or cured it. Similarly Morgan ('34) showed that acid diets intensified the degree of rickets in dogs. However, the exact effect of alkali is not clear from her data. In any event the basal diet apparently was not only rachitogenic, but sufficiently so that with the amount of cod liver oil she used it was not clear that healing took place. So far as we are aware, we produce here the first clear evidence that changes in acid-base alone can convert a non-rickets producing diet into a rickets producing diet or vice versa. Moreover the result is definite, not for a single diet but for all types of diet in which calcium and phosphorus are the main variables.

That the addition of NH_4Cl alone produces rickets indicates that the Hamilton thesis, that alkaline reaction in the intestinal tract is a necessary factor, has not been confirmed. That $(NH_4)_2CO_3$ enhances the effect is, however, evidence that the alkaline reaction is a supporting factor.

It is further clear that the combination of acid reaction with alkaline ash is not alone responsible for rickets prevention in diets which are rachitogenic to this degree because it is effective only when tartrates or citrates are used. From our study of the citrates, however, it is shown that neither an acid reaction, nor an alkaline ash alone is sufficient and that both are necessary to prevent rickets in these diets. That

orange juice increased the retentions of Ca, P, Mg and N in children was shown by Chaney and Blunt ('25). The beneficial effect was attributed to the citric acid-alkaline citrate. The significance of these findings as applied to rickets was emphasized by Querido ('35).

Morris and MacRae ('32) gave NH_4Cl to infants with healing (?) rickets plus tetany and reported that calcification was not prevented. Acid milk has been used to cure rickets, but unless the acid is in too great an excess this should simulate the conditions of increased acid reaction in the ingested food and a mineral residue which is still alkaline. However, Hess and Matzner ('24) stated that acid milk did not cure rickets. Zucker's ('22) original experiments showed that acid added to the diet rendered it less rickets producing. However, he informed the author personally that unpublished data showed that when the acid was increased beyond the neutral point the degree of rickets was then again increased.

In the present study, the diets used varied in acidity as shown in table 2. Those with the most $CaCO_3$ were most alkaline and those with KH_2PO_4 were most acid. It can be argued that the experimental diets should all be neutral. Such a plan was devised by the use of acid and alkaline phosphate mixtures, and of $CaCO_3$-$CaCl_2$ mixtures. The reasons why such diets were not used were discussed in the previous paper (Shohl, '36). Such a regime might give a different curve for the border line of rickets production, but a new set of variables thus introduced would prevent comparison with our previous results. However, our present conclusions would not be altered, but could only be more exactly defined because the effect was shown with acid and alkaline as well as neutral diets.

From the per cent ash one cannot differentiate between osteoporosis and rickets; the value becomes a comparative measure only when the lesions are known to be similar—i.e., by histologic examination. This is obvious from inspection of the data on the animals fed lactates and tartrates, shown in table 3. The bones of the former showed a greater per cent

of ash than the latter, but the animals receiving lactates had rickets and those receiving tartrates had none.

Because no light has been thrown on the mechanism of rickets production or prevention by either growth or blood studies it seems that metabolic studies are necessary to determine how these results are brought about, and how the metabolism of Ca and P are altered. In light of the well-known property of citrate solutions to form complex calcium ions, as demonstrated by Hastings et al. ('34) an attractive field for further investigation is suggested.

By the addition of salt mixtures to make diets acid in reaction and alkaline in ash or vice versa the acid-base effects of diets on the production and prevention of rickets have been brought out more clearly than ever before.

Alterations in the Ca and P intake have previously been shown to cure experimental rickets. The addition of citrates, either alone or in conjunction with Ca and P additions without vitamin D or ultraviolet light, offers a second method for possible therapeutic application to infantile rickets.

SUMMARY AND CONCLUSIONS

The diets used were made to contain eight different ratios of calcium to phosphorus. In each of these ratios there are zones where rickets were produced when the absolute amounts were low and no rickets when the amounts were increased.

1. a. The addition of NH_4Cl–$(NH_4)_2CO_3$ mixtures to non-rachitogenic diets, in each of the eight zones, renders them rachitogenic. Further, the same additions to the rachitogenic diets in each of the eight zones, intensifies the severity of the rickets produced.

b. The NH_4Cl is the more important moiety, though the $(NH_4)_2CO_3$ enhances the effect.

2. a. The addition of citric acid-sodium citrate mixtures to rachitogenic diets in each of the eight zones alters them so that they no longer produce rickets.

b. Both the acid reaction and the alkali ash factors are necessary to accomplish this result.

c. In the prevention of rickets, the citrates and tartrates alone, among the organic acids tested, were effective; therefore their action cannot depend entirely upon their acid-base effects.

It is a pleasure to acknowledge the assistance of Dr. S. B. Wolbach for the histological interpretation of the bones, of Miss E. MacLachlan for the Ca and P determinations and of Dr. E. Vogt for the x-ray pictures and assistance in their interpretation.

LITERATURE CITED

BROWN, H. B., A. T. SHOHL, E. E. CHAPMAN, C. S. ROSE AND E. M. SAURWEIN 1932 Rickets in rats. XIII. The effect of various levels and ratios of calcium to phosphorus in the diet upon the production of rickets. J. Biol. Chem., vol. 98, pp. 207–214.

CHANEY, M. S. AND H. BLUNT 1925 The effect of orange juice on the calcium, phosphorus, magnesium and nitrogen retention and urinary organic acids of growing children. J. Biol. Chem., vol. 66, pp. 829–845.

FISKE, C. H. AND M. A. LOGAN 1931 The determination of calcium by alkalimetric titration. J. Biol. Chem., vol. 93, pp. 211–226.

FISKE, C. H. AND Y. SUBBAROW 1925 The colorimetric determination of phosphorus. J. Biol. Chem., vol. 66, pp. 375–400.

FREUDENBERG, E. AND P. GYÖRGY 1922 Die pathogenetischen Beziehungen zwischen Tetanie und Rachitis. Munch. Med. Wchnschr., Bd. 69, S. 422–423.

GOLDBLATT, H. 1931 Die neuere Richtung der Experimentellen Rachitisforschung. Ergebnisse allgem. Path. path. Anat. Menschen Tiere, Bd. 25, S. 58–491.

GYÖRGY, P. 1929 Die Behandlung und Verhütung Rachitis und Tetanie, nebst Bemerkungen zu ihrer Pathogenese und Aetiologie. Berlin, S. 221.

HAMILTON, B. AND C. SCHWARTZ 1933 Role of acidosis and alkalosis in the etiology of rickets. Am. J. Dis. Child., vol. 46, pp. 669–670.

HASTINGS, A. B., F. C. MCLEAN, L. EICHELBERGER, J. L. HALL AND E. DA COSTA 1934 The ionization of calcium, magnesium and strontium citrates. J. Biol. Chem., vol. 107, pp. 351–370.

HESS, A. F. 1929 Rickets including osteomalacia and tetany. Philadelphia, p. 167.

HESS, A. F. AND M. J. MATZNER 1924 Value of milk acidified with lemon juice; its combination with egg yolk to add the antirachitic factor. J. Am. Med. Assoc., vol. 82, pp. 1604–1606.

MORGAN, A. F. AND OTHERS 1934 The effect of acid, neutral and basic diets on the calcium and phosphorus metabolism of dogs. Univ. Calif. Pub. Physiol., vol. 8, pp. 61–106.

MORRIS, N., F. J. FORD AND S. GRAHAM 1936 The role of acidosis and phosphate retention in pathogenesis of rickets and rachitic tetany of infants. Acta Paediatrica, vol. 18, pp. 50–91.

MORRIS, N. AND O. MACRAE 1932 Effect of ammonium chloride administration on metabolism in infantile tetany. Arch. Dis. Child., vol. 7, pp. 47–58.

QUERIDO, A. 1935 Vitamin D requirements in relation to the Ca and P content of the diet. Archiv. Néerland. Physiol. de l'homme et ses animaux, vol. 30, pp. 487–538.

SCHLOSS, E. 1917 Die Pathologie und Aetiologie der Rachitis sowie die Grundlagen ihrer Therapie. Ergeb. inn. Med. u. Kinderh., Bd. 15, S. 55–138.

SHELLING, D. H. 1925 The production of ricket in rats by changes in the acid-base ratio of the diet. Thesis, Yale University, 63 pp.

SHOHL, A. T. 1936 Rickets in rats. XV. The effect of low calcium-high phosphorus diets at various levels and ratios upon the production of rickets and tetany. J. Nutrition, vol. 11, pp. 275–291.

SHOHL, A. T., H. B. BENNETT AND K. L. WEED 1928 Rickets in rats. IV. The effect of varying the acid-base content of the diet. J. Biol. Chem., vol. 78, pp. 181–190.

SHOHL, A. T., H. B. BROWN, C. S. ROSE, D. N. SMITH AND F. COZAD 1931 Rickets in rats. XII. The acid-base equilibrium of the blood in rickets and tetany. J. Biol. Chem., vol. 92, pp. 711–719.

SHOHL, A. T., H. B. BROWN, E. E. CHAPMAN, C. S. ROSE AND E. M. SAURWEIN 1932 Rickets in rats. XIV. A diet which demonstrates the effect of the acid-base content upon the production of rickets and also causes idiopathic tetany. J. Biol. Chem., vol. 98, pp. 215–224.

ZUCKER, T. F., W. C. JOHNSON AND M. BARNETT 1922 The acid-base ratio of the diet in rickets production. Proc. Soc. Exp. Biol. and Med., vol. 20, pp. 20.

SYMPTOMATOLOGY AND PATHOLOGY OF POTASSIUM AND MAGNESIUM DEFICIENCIES IN THE RAT

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TWO PLATES (EIGHT FIGURES)

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Although it has long been recognized that potassium and magnesium are essential for animal and plant life, the effect of a deficiency of these elements on animals has been studied only recently. Information concerning the histopathology of potassium or magnesium deficiency is especially scanty.

Very little work has been done on the symptomatology of potassium deficiency. Miller ('23) reported a very slow growth and an abnormal alertness as the two principal symptoms of rats fed a synthetic diet low in potassium. Leulier and Vanhems ('34) found that young rats failed to grow and died in 12 to 24 days when fed a diet deficient only in potassium. We were unable to find any reports on the histopathology of potassium deficiency.

The symptomatology of magnesium deficiency has received considerable attention in recent years, probably due largely to the excellent reports of McCollum and Orent ('31) and of Kruse, Orent and McCollum ('32 a) on the rat, and of Orent, Kruse and McCollum ('32) on the dog. Leroy ('26) had earlier reported that mice died in 24 to 35 days, when fed a low magnesium diet, but mentioned no other symptoms. In the main the findings of the above workers on the symptomatology in magnesium deficiency of the rat have been confirmed by Brookfield ('34). Greenberg and Tufts ('35 a, '35 b)

produced only the symptoms of convulsions in rats on a low magnesium diet. Moreover, the convulsive seizure was not spontaneous, but had to be induced by a suitable stimulus, and could not be induced if the animals received sufficient vitamin G. They also found that the time required for the appearance of convulsions was dependent on the vitamin G intake. Cramer ('32) noticed no effect on the health of rats on a low magnesium diet, during a 6-week experimental period.

Recent work indicates that, under certain conditions, symptoms of magnesium deficiency may develop in livestock. A peculiar disease called 'grass tetany' is known to occur in dairy cows. The etiology of the disease is obscure, but it has been shown (Sjollema, '32 a, '32 b, and Sjollema and Seekles, '32) that a very low blood magnesium is present. The symptoms of the disease strongly resemble those of magnesium deficiency and the above workers and also Pulles ('31) found that injections of a mixture of $CaCl_2$ and $MgCl_2$, if done in time, would cure the disease. Following the reports by Kruse, Orent and McCollum ('32a), Sjollema ('32 c) reached the conclusion that 'grass tetany' is primarily caused by a low magnesium level in the blood. Green, Allcroft and Montgomerie ('35) reported a low blood magnesium in equine transit tetany and considered this disease to be similar to 'grass tetany' in cows. Calves, which have been reared mainly on a milk diet, may develop magnesium deficiency, as evidenced by a low plasma magnesium and tetany (Duncan, Huffman and Robinson, '35). Huffman and Duncan ('36) later found that both the low plasma magnesium and the tetany in calves were prevented by feeding magnesium in the form of the oxide, the carbonate or natural foods.

Although it is highly improbable that the average diet of humans is low in magnesium, apparently certain occasions may arise when the intake of this element is insufficient. Hirschfelder ('33 and '34) has reported a low blood magnesium in ten patients, which was accompanied by recognizable symptoms of magnesium deficiency; these patients were receiving special soft diets, which included milk; oral administration of $MgSO_4$ to three of the patients resulted in a

rise in blood magnesium and a disappearance of convulsions. Daniels and Everson ('36) conducted mineral metabolism experiments on pre-school children who received complete diets. It would appear possible, from their analytical data on urinary magnesium, that some of the children may have been on a previous dietary of sub-optimum magnesium content.

The pathology of magnesium deficiency has received some attention. Kidney lesions were found by Cramer ('32) in rats fed a low magnesium diet. Brookfield ('34) reported degenerative changes in the liver as well as in the kidney of the rat. Moore, Sholl and Hallman ('36) recently reported the pathology associated with a low blood magnesium in dairy calves. They found that "in general the microscopic picture involved a basophylic hyaline-like necrosis of the collagenous and yellow elastic connective tissue elements of the heart, blood vessels, spleen, peritoneal and pleural surfaces of the diaphragm." Chemical changes in the blood (Kruse, Orent and McCollum, '32 b and '33), in the bone (Orent, Kruse and McCollum, '34), and in the mineral metabolism (Kruse, Schmidt and McCollum, '34) have also been reported.

During a study of vitamin G deficiency in the rat and the chick in this laboratory, diet 231 of Keenan, Kline, Elvehjem and Hart ('35) was used; atypical symptoms were encountered when the yellow corn, wheat middlings, and casein were extracted with dilute acetic acid instead of being heated. These symptoms were traced to a deficiency in the diet of both potassium and magnesium. Interesting symptomatology and pathology were observed in rats in each instance. A search of the literature revealed scanty information concerning potassium deficiency, and contradictory findings in magnesium deficiency. Because of these facts, a somewhat detailed study of each deficiency has been made on the rat. The results are presented in the following pages.

EXPERIMENTAL PROCEDURE

The rats were started on experiment at an average weight of 55 gm. Their average age was 24 days, with a range of from 22 to 25 days. Individual metal cages were used in all experiments. All rats received the basal diet ad libitum; the food was weighed out daily. Distilled water was always available. Some groups received their respective basal diets only; others received the basal diet plus added vitamin B. The vitamin B supplement was a fuller's earth adsorbate of a brewer's yeast extract. Ten milligrams of adsorbate was given on alternate days. Pigeon tests showed the above quantity to contain 3 international units of vitamin B (Salmon and Goodman, '34).

The same basal diet was used for all the feeding experiments; it varied only in the inorganic supplements. These were added, when necessary, at the expense of the extracted grain mixture. Essentially this diet was the same as diet 231 which had been used by Keenan, Kline, Elvehjem and Hart ('35). It differed only in the treatment of the yellow corn, wheat middlings, and casein. Instead of heating these constituents they were subjected to extraction. The casein was extracted as described by Salmon and Goodman ('34). About 40 pounds of the grain mixture, consisting of 59 parts ground yellow corn and 25 parts wheat middlings, was extracted by percolation in a large monel metal kettle with 0.2% acetic acid for 6 days; tap water was used the first 5 days and distilled water the sixth day. Distilled water alone was used the seventh day. On the eighth day the extracted grain mixture was pressed as dry as possible in a filter press, and was placed in shallow enamel pans and dried in a forced draft oven at 60 to 70°C.; it was then ground to the desired degree of fineness. The diets were made up about every 10 days. When not in use they were stored in a cold room at a temperature of about 3°C.

The diets were fortified with salt mixture no. 186 (Guerrant and Salmon, '30), or with simple salts of C.P. grade. Magnesium, in the form of the trihydrated sulfate, and potassium, in the form of the chloride, were added in such amounts

as would furnish the same quantity of magnesium and potassium as were contained in 4% of salt mixture no. 186. The chloride content of the diets containing simple salts was maintained at a constant level. The composition of the basal diets is given in table 1.

Besides the rats on the potassium and the magnesium deficient diets (231E + Mg and 231E + K, respectively), a group of rats was fed a diet deficient in both potassium and magnesium (231E). This group served as a negative control.

TABLE 1

Percentage composition of basal diets

INGREDIENTS	231E	231E + MG	231E + K	231E + MG + K	232
Extracted grain mixture [1]	84.0	83.5	82.6	82.1	83.0
Extracted casein [2]	12.0	12.0	12.0	12.0	12.0
Cod liver oil	1.0	1.0	1.0	1.0	1.0
Salts 186 [3]	....	....	....	....	4.0
NaCl	1.0	1.0	....	....	....
$CaCO_3$	1.0	1.0	1.0	1.0	....
$Ca_3(PO_4)_2$	1.0	1.0	1.0	1.0	....
$MgSO_4 \cdot 3H_2O$	....	0.5[4]	....	0.5[4]	....
KCl	....	....	1.0[5]	1.0[5]	....
$NaHCO_3$	....	....	1.4[6]	1.4[6]	....

[1] Ground yellow corn 59 parts and wheat middlings 25 parts. This mixture was extracted with 0.2% acetic acid for 6 days and distilled water 1 day.

[2] J. Nutrition, '34, vol. 8, p. 1.

[3] J. Biol. Chem., '30, vol. 89, p. 199.

[4] This quantity of $MgSO_4 \cdot 3H_2O$ furnishes the same amount of Mg as is supplied by 4% of salts 186.

[5] This quantity of KCl furnishes the same amount of K as is supplied by 4% of salts 186.

[6] This quantity of $NaHCO_3$ furnishes the same amount of Na as is supplied by 1% of NaCl.

A positive control group received both potassium and magnesium in the form of the simple salts (diet 231E + Mg + K). As an additional positive control, a group of rats was fed the basal grain mixture supplemented with the complex salt mixture no. 186 (diet 232).

With the exception of a few cases in which tissues showing the various stages of the skin lesions found in animals on the

magnesium deficient diet were wanted for microscopic study, the animals on the deficient diets were allowed to continue on experiment until moribund. Control animals were killed at comparable periods. When necessary, the animals were killed with chloroform. Table 2 shows the number of animals on which necropsies were made, and also the number used for histopathological studies.

The tissues used for microscopic study represent a cross section of the groups on which gross observations were made. In the case of the groups on the magnesium deficient diet, only the tissues of animals known to have died in convulsive seizures were used. More time and more animals were not devoted to this phase of the work because within any given group the findings were remarkably consistent. The tissues were removed immediately after death, fixed in 10% formol-saline, embedded in Parlodion and sectioned at 15 μ. They were stained with Harris' hematoxylin and counterstained with either eosin Y or Triosin.[1]

RESULTS

Potassium deficiency

Growth and duration of life. The rats on the potassium deficient diet made practically no growth, and at death had lost an average of about 10% of the starting weight (table 2). The average time to death or closing out was 23 days. Vitamin B addition had no significant effect on weight or length of life.

Symptomatology. Early in the experiment, the rats began to show a lethargic condition which gradually progressed into coma and death. Accompanying the lethargic symptoms was a progressive degree of abdominal distention which was usually very marked by the time the animals became moribund. The skin was pale and somewhat cyanotic; the hair was short and fur-like. In a few cases, pendulous sacs were seen in the region of the thyroid gland and the manubrium of the sternum.

[1] Triosin is a differential plasma stain manufactured by Albert E. Galigher, Inc., 1228 Solano Avenue, Berkeley, California. In our experience, it has proved more selective than eosin.

Gross pathology. At necropsy, a tremendous amount of pathology was found, the abdominal cavity being the site of the greatest amount of change. Severe ascites was usually present, there often being as much as 4 to 5 cc. of clear straw-colored fluid. In a few cases, hydrothorax and hydropericardium were noted.

TABLE 2

Average growth and duration of life of rats on the K and Mg deficient diets, together with the number of rats used for gross and histopathological studies

BASAL DIET	SUPPLEMENT	GROWTH STUDIES				PATHOLOGY STUDIES	
		Number of rats	Maximum increase in weight	Final change in weight [1]	Time to death or closing out	Rats used for gross pathology	Rats used for histopathology
			gm.	*gm.*	*days*		
231E	None	12	0	—6	26	7	4
231E	Vitamin B [2]	10	0	—7	23	10	5
231E + Mg	None	11	0	—6	23	10	5
231E + Mg	Vitamin B [2]	18	1	—5	21	8	5
231E + K	None	25	11	5	34	16	10
231E + K	Vitamin B [2]	24	17	12	37	15	8
231E + Mg + K	None	6	47	47	56 [3]	18 [4]	4
231E + Mg + K	Vitamin B [2]	6	48	48	56 [3]	18 [5]	4
232	None	6	53	53	56 [3]	6	..
232	Vitamin B [2]	5	49	49	56 [3]	5	..

[1] The difference between the average starting weight and the final weight at death or closing out.

[2] Ten milligrams of a fuller's earth adsorbate of a brewer's yeast extract given on alternate days to each rat. Pigeon tests showed this quantity to contain 3 I.U. of vitamin B.

[3] No deaths occurred in any of these four groups.

[4] Six rats were killed at 21 days, six at 33 days, and six at 56 days. The former two groups of six served as controls for groups 231E + Mg and 231E + K, respectively.

[5] Six rats were killed at 21 days, six at 36 days, and six at 56 days. The former two groups of six served as controls for groups 231E + Mg + vitamin B and 231E + K + vitamin B, respectively.

The intestinal tract was tremendously enlarged, markedly atonic, and had a translucent agate-like appearance. The ileum and lower jejunum were the site of the severest changes but, in a few cases, the whole intestinal tract was affected. In the ileum, thickened annular areas were observed, usually

surrounding Peyer's patches; these thickenings gave the intestine a 'beaded' appearance. In such areas the congestion was often so marked that it was thought to be hemorrhage. Intervening portions of the intestinal tract appeared anemic and were often collapsed.

In the predominating number of animals, intussusceptions were found in the ileum and lower jejunum. As many as four were observed in a single animal. In one case, a large intussusception involving the major portion of the ileum had entered the cecal pouch. No gross evidence of infarction of the intestine was noted.

The mesenteric lymph nodes were markedly enlarged, translucent, and surrounded by opalescent jelly-like material. Accumulations of this material were also observed in the pendulous sacs in the region of the thyroid and also in the axilla. The kidneys were enlarged and very pale; when they were sectioned longitudinally, fluid was seen to exude from the cut surface. Commonly, there was a marked congestion of the suprarenal glands and, in one case, hemorrhage. The liver was somewhat pale and appeared flabby.

The pancreas was usually markedly dissociated into its constituent lobules which were loosely bound together by opalescent jelly-like material. Often the scattered pancreatic tissue had a distribution similar to that of the greater omentum.

On the lateral aspect of the right ventricle of the heart, at the apex and, less frequently, on the left ventricle were noted opaque areas of variable size.

Necropsy findings on animals on the potassium deficient diet supplemented with vitamin B differed from those not receiving this supplement in only one particular. In these animals, intussusceptions were found to be a much less frequent occurrence.

Microscopic pathology. The most consistent and most severe changes were found in the lower jejunum and ileum. However, in a few cases the stomach, duodenum and upper jejunum were the site of mild effects. Two distinct types of change were observed which corresponded in position to the

changes noted at necropsy. In areas corresponding to the enlarged, annular, or 'beaded' portions of the intestine, the Peyer's patches were usually markedly congested, edematous, rarefied and contained numerous mononuclear phagocytic cells. No hemorrhages were noted. The villi were seen as apparently confluent, distended bodies which contained innumerable lymphocytes and phagocytes (plate 1, B). The mucosa, particularly at the tips of the villi, was markedly edematous, vacuolated, and the nuclei of the columnar cells were usually pycnotic. Frequently the tip of the villus was the site of an accumulation of phagocytes in various stages of degeneration which formed a 'cap.' Sloughing of portions of the mucosa with apparent rupture of the villi which allowed its contained lymphocytes and phagocytes to enter the intestinal lumen was occasionally seen. Numerous phagocytic cells, both mononuclear and polymorphonuclear, were consistently found within the lumen of the intestine in this region. They were apparently embedded in the jelly-like material observed at necropsy. The borders of this type of lesion were sharply defined.

The second type of lesion corresponding in position to the tissue intervening between the 'beaded' portions showed more of an edematous change. The vascular elements were markedly congested. The submucosa as a whole was usually very rarefied, edematous, and distended, but frequently the distention was confined to the tips of the villi giving them a 'clubbed' appearance (plate 1, A, C). The mucosal epithelium was usually shortened and thickened, appeared atrophic, and the nuclei were pycnotic. However, the columnar cells at the tips of the villi were frequently edematous and vacuolated, and were 'capped' by accumulations of phagocytes. The lumen of the intestine in the region of this type of change contained few phagocytic cells.

The large intestine occasionally showed mild edema in the submucosa and congestion of blood vessels. The mesenteric lymph nodes were enlarged, edematous, rarefied and contained considerable numbers of mononuclear phagocytes.

In the portions of the intestines involved in intussusceptions, the same changes were observed as in other portions of the lower jejunum or ileum, but no evidence of infarction was noted.

Massive erosions involving both the endocardium and the myocardium were found in both ventricles of the heart. These lesions varied in size and had rather sharply defined borders. There was usually complete destruction of cardiac musculature and replacement with scar tissue and numerous phagocytes (plate 1, D). In several cases, the erosion had almost perforated the heart wall. There was usually a variable amount of interfibrillar infiltration of lymphocytes in the tissues in close proximity to the erosions. The cardiac vessels were usually engorged. In a few cases, whole muscle bands stained deeply, the nuclei appeared pycnotic, and the cells shrunken. Other muscle bands on either side, if not involved by the erosive type of lesion, were apparently normal. Occasionally, the erosions were noted in the valvular tissue, but no changes were ever found in the auricles.

Considerable engorgement of blood vessels and foaminess of hepatic cells were the only findings in the liver.

The pancreas was usually markedly dissociated into fragments of variable size embedded in the jelly-like mass. The interlobular connective tissue was practically absent. The sections stained poorly, being homogeneous and hazy in appearance. Acinar tissue appeared edematous. In a few cases, the number of islands of Langerhans seemed diminished and those remaining appeared somewhat edematous and hypertrophic. Numerous phagocytes were usually seen in the jelly-like material.

Diffuse tubular nephritis was present in all kidneys observed (plate 2, A). The epithelium of the tubules was markedly edematous, vacuolated, and had sloughed in some cases. Pycnosis of nuclei was common. There was a considerable degree of congestion of the renal blood vessels. Little change was seen in the glomeruli.

In the suprarenals, there was marked engorgement of blood vessels. The cortical cells stained poorly and, in a few cases, appeared rarefied and edematous.

The tissues of animals which had received the diet containing an adequate amount of potassium showed no microscopic changes.

Magnesium deficiency

Growth and duration of life. The rats on the magnesium deficient diet gained an average of about 10 gm., mostly in the first 3 weeks on experiment. Some of the gain in weight was lost by the time death occurred at an average time of 34 days (table 2).

Symptomatology. The animals began to show mild symptoms of hyperirritability as early as the end of the first week on experiment. This hyperirritability became more marked as the experiment continued.

About the end of the first week on experiment a generalized hyperemia of the skin was observed, which was succeeded about the tenth day by an erythema which was most severe on the dorsum of the back and on the flanks. In some cases, the erythematous change was observed in the scapular, cranial, masseteric, and mandibular regions, and at the roots of the ears. Although the erythema was occasionally generalized, it was most often present in discrete circular areas of variable size. Intervening skin was apparently normal. Occasionally, pronounced edema was seen in the pedal portion of the posterior extremities and in the nasal region.

Succeeding the erythema the greater number of animals developed more or less extensive purpural skin hemorrhages. They were confined to the areas which had shown erythema. Usually a variable amount of eschar formation followed the purpura. The eschars were both of the dry and moist type, and were found most consistently on the back in the sacral region and on the flanks. However, they were also observed in a few cases in the scapular, cranial, masseteric, and mandibular regions. They were occasionally seen as confluent masses of escharotic tissue but were more often discrete

and circumscribed (plate 2, B). Following the escharotic stage, exfoliation and healing took place leaving the skin mildly scaly and the area denuded. The duration of the escharotic stage was extremely variable, occasionally lasting throughout the remainder of the experiment. The purpural and escharotic changes were not necessarily a sequence to the erythema, but were never observed unless the animal had previously shown it.

Tonic-clonic spasms were observed as early as the seventeenth day, and as late as the fifty-first day. The average time of onset was 30 days. The symptomatology of these seizures was essentially the same as that described by Kruse, Orent and McCollum ('32 a) with the following exception: in only one animal was more than one seizure noted during an attack. This animal had three severe seizures in succession.

Death was characterized by its suddenness and its unpredictability. However, the fact that most animals were found in positions similar to those observed during convulsive seizures is suggestive that death followed such an experience.

Gross pathology. At necropsy, few pathological changes could be found in the gross. Occasionally, pigmentation and apparent scarring of the skin were observed in areas which had previously been affected by the purpural and escharotic lesions. The intestinal tract was mildly atonic, congested and, in a few cases, mild gaseous enteritis was seen. The suprarenal glands were usually congested. The heart appeared somewhat enlarged and flabby.

In the greater number of animals necropsied, the liver appeared somewhat pale, mottled, and flabby, but in nine animals massive lesions were observed. These changes were of two types. The first type, which was found in five animals, consisted of dark red areas in which the lobulation of the liver appeared as in mosaic. The borders of these lesions were discrete and circumscribed, and apparently had no particular site of distribution. In the second type, found in two animals, the livers were markedly contracted by extensive cicatrizing

lesions which sent out finger-like projections of scar tissue deep into the liver substance. They were apparently an older form of change than the mosaic type. In two cases, both the cicatrizing and the mosaic type of change were observed in the same liver.

Animals on this same diet supplemented with vitamin B showed no significant difference from those not receiving this supplement.

Microscopic pathology. Considering the violent nature of death in rats on this diet, very little pathological change was found when the microscopic examination of the tissues was made.

The intestinal tract evidenced only a mild congestion of the vascular elements. The heart was normal as was the pancreas. In the kidneys occasional tubules showed mild degenerative changes. There was no calcification. The suprarenals were markedly congested.

Sections of the skin in the active erythematous stage showed only marked engorgement of blood vessels and edema. In the escharotic stage, there was edema, mild infiltration of white blood cells, and at times sloughing of the cuticular layer. In sections of skin taken when all gross evidence of the lesion had disappeared, there was generalized sloughing of the cuticular layer and, in some cases, scarring.

In the greater number of livers examined, mild foaminess of the cellular elements and marked congestion of the vascular tissues were the only changes noted. However, in the livers which had shown the massive cicatrizing lesions at necropsy, the changes were diffuse and varied. Practically all liver cells were affected to some extent. Vascular proliferation and perivascular accumulations of small round cells were common. Numerous areas of scar tissue accompanied by marked infiltration of lymphocytes and phagocytes were seen (plate 2, C). In some places the tissues were practically acellular, and had a homogeneous hyaline-like appearance.

The second type of lesion in which, at necropsy, the lobulation of the liver was seen as if in mosaic, appeared to be a

recent involvement. No scarring was observed, but vascular proliferation and perivascular infiltration of small round cells were common. In most areas, the damage was confined to the periportal regions whereas in others it was diffuse (plate 2, D). The degenerated areas were practically acellular except for a few pycnotic nuclei and numerous phagocytes containing green-yellow ingested material. Occasionally, gigantic nuclei were observed which appeared normal in structure. The borders of the lesions were rather sharply defined, but all liver cells showed a marked foaminess of the cytoplasm.

The tissues of animals which had received the diet containing an adequate amount of magnesium showed no microscopic changes.

Magnesium and potassium deficiency

The rats on the diet which was deficient in both potassium and magnesium resembled the potassium deficient rats in so far as change in weight and time to death were concerned (table 2). They evidenced a considerable degree of hyperirritability in the early part of the experiment, which corresponded to that observed in animals on the magnesium deficient diet. In contrast to the animals on the magnesium deficient diet, however, the hyperirritability soon disappeared and the animals began to show more of the lethargic type of symptoms seen in animals on the potassium deficient diet.

No skin lesions or erythema were observed in these animals. They became progressively more lethargic and weakened; abdominal distention became marked and, at the time of death, they were usually in a comatose condition.

Necropsy and microscopic findings in this group were essentially the same as those observed in animals on the potassium deficient diet and will not be repeated here. The addition of vitamin B had no apparent effect.

Positive controls

No deaths occurred, either in those rats receiving the simple potassium and magnesium salts or in those receiving the complex salt mixture, during an 8-week experimental period. A

steady gain in weight was maintained, which was practically identical in both groups. The addition of vitamin B had no appreciable effect on the results obtained from either group. The data are summarized in table 2. Similarly, no gross pathological changes were observed in either group. Due to the lack of gross pathology, microscopic studies of tissues of rats which received the complex salt mixture were not made.

DISCUSSION

The important role that potassium plays in animal metabolism is dramatically illustrated by the short duration of life and the pronounced tissue changes occurring in young rats fed a diet deficient in this element. The duration of life and effect on growth obtained by Leulier and Vanhems ('34) are in substantial agreement with our data. They did not mention any other symptoms, however. Our rats developed short fur-like hair, cyanosis, abdominal distention, and lethargy leading to coma and death. It is possible that Miller's diet ('23) was not as low in potassium as the analyses indicated because his rats did not die during a 17-week experimental period. In contrast to the abnormal alertness of his rats, our animals exhibited a lethargic condition. His rats made a slow gain in weight; our rats lost weight slowly from the beginning of the experiment. The fact that the rats on our magnesium deficient diet showed an initial gain in weight is further proof of the extreme importance of potassium in animal nutrition.

Many of the pathological changes such as ascites, hydrothorax, hydropericardium, and the marked edematous effects observed in the intestines of the rats on the potassium deficient diet would tend to indicate a pronounced disturbance of fluid metabolism. Such a result might be expected from a lack of an element so intimately concerned with the fluid balance of the body.

The marked atonicity, ptosis, and, at times, the almost complete absence of peristaltic movements observed in the lower jejunum and ileum would tend to support the work of Robert-

son and Doyle ('35) on intestinal stasis in rats, which suggested to them that constipation may be a result of too low a level of potassium intake.

Whether or not the intussusceptions noted in our experiments were a part of the pathological findings due specifically to potassium deficiency is a question, but they would serve to indicate that peristalsis was impaired. The fewer intussusceptions found in the potassium deficient rats which received vitamin B may indicate that this condition was not a specific characteristic of the deficiency. There is the possibility, however, that the vitamin B solid carried sufficient potassium to partially prevent their occurrence. We have found no intussusceptions in hundreds of rats showing the extreme incoordination of acute vitamin B deficiency produced by diets containing adequate potassium. Moreover, the potassium deficient diet used in the present investigation contained ample vitamin B for the rat despite the extraction with acidulated water.

The early symptoms produced in young rats fed the magnesium deficient diet are essentially in agreement with those reported by McCollum and Orent ('31) and by Kruse, Orent and McCollum ('32 a) for the same animal. Certain differences should be mentioned, however. Their animals exhibited the following effect on the skin: "Within 3 to 5 days, average 4 days, all the exposed skin areas became vividly red from vasodilatation and hyperemia in the vascular bed." Our animals consistently developed a generalized hyperemia or vasodilatation of the skin. This condition was usually followed by the formation of circumscribed areas of erythema, hemorrhages, eschars, and exfoliation, especially prevalent on the dorsum of the back and on the flanks. In addition, there was occasionally seen in the later stages a pronounced edema in the pedal portions of the posterior extremities and in the nasal region. Greenberg and Tufts ('35 a, b), Cramer ('32), and Brookfield ('34) apparently obtained none of these symptoms in rats.

In general, our findings agree with those of Kruse et al. ('32 a) and Brookfield ('34) in regard to the tonic-clonic convulsive seizures and death. Our rats, however, usually exhibited only one seizure during an attack and did not always develop a convulsion when stimulated. Although some animals needed only a slight stimulus to induce a seizure, others required a prolonged stimulus; still other rats apparently passed into a seizure spontaneously. However, the greater number of animals died during the night. The postures assumed by them suggested that death may have resulted from a convulsion. Cramer ('32) observed no convulsions in rats fed a low magnesium diet, and Greenberg and Tufts ('35 a, b) obtained a convulsive seizure only after drastic stimuli were provided. The latter workers claim that increasing the vitamin G content of the diet entirely prevented the convulsive seizures.

Undoubtedly our basal diet had lost a good deal of its vitamin G content as a result of the prolonged extractive procedure used in its preparation. The group of rats on diet 232 plus vitamin B (table 2) was kept on experiment a total of 13 weeks. No abnormality ever appeared except the continued subnormal rate of growth; the rats averaged 121 gm. in weight at this time. This retarded growth may have been due to a suboptimum intake of vitamin G or it may have been due to a deficiency of other water-soluble factors.[2] The difficulty in obtaining magnesium or potassium free sources of the water-soluble vitamins should be considered in all work dealing with a deficiency of these elements. However, the fact that either the simple potassium and magnesium salts or the complex salt mixture prevented all the symptomatology and pathology was strong proof that these conditions in our

[2] The lacking factors were mainly supplied by brewer's yeast. Diet 232 supplemented with 0.5 gm. of brewer's yeast per rat daily gave a near normal gain; two female and two male rats had an average weight of 222 gm. after 8 weeks on experiment.

experiments were due to a deficiency of potassium or magnesium,[3] as the case might be.

Considering the spectacular nature of the symptoms obtained during magnesium deficiency in our rats, surprisingly little histopathology was encountered. Aside from the usual occurrence of changes in the skin, one-third of the animals studied microscopically (nine out of twenty-seven) evidenced degenerative changes in the livers. Just how much significance this liver degeneration has in magnesium deficiency is unknown. However, Brookfield ('34) also reported liver degeneration, but we are unable to determine from his data whether the same type of change was produced. Our findings are in disagreement with those of Brookfield ('34) and Cramer ('32) in showing no degeneration in the kidneys.

SUMMARY

1. Rats receiving a potassium deficient diet showed slow loss in weight, short fur-like hair, cyanosis, abdominal distention, and lethargy leading to coma and death (23 days average). Pathological changes occurred in the intestine, pancreas, kidneys and heart. Marked ascites was usually present. Hydrothorax and hydropericardium were an occasional occurrence.

2. Rats receiving a magnesium deficient diet showed a very subnormal growth in the early part of the experiment; part of this gain in weight was lost before the animals died. The animals consistently developed generalized hyperemia of the skin, which was followed by circumscribed areas of erythema, hemorrhages, eschar formation, and exfoliation. Edema of the extremities and nasal region was an occasional finding. An early hyperirritability, apparently progressive, led to

[3] Additional proof of the cause of the symptoms produced in rats fed the magnesium deficient diet (231E + K) was obtained. This diet was low in inorganic sulfur as well as in magnesium, because the former was supplied entirely as sulfate in $MgSO_4$. Sulfur in the same form, Na_2SO_4 in this case, was added to diet 231E + K in the same amount as was furnished by 4% of salts no. 186 or by 0.5% of $MgSO_4 \cdot 3H_2O$. The sodium content of the diet was maintained at a constant level by reducing the amount of $NaHCO_3$. No beneficial effect was observed on six rats fed this sulfur-supplemented magnesium deficient diet.

tonic-clonic convulsions (30 days average), and often to death (35 days average). Death was characterized by its suddenness and unpredictability. Considering the spectacular nature of the symptoms, surprisingly little histopathology was encountered. Aside from the usual occurrence of changes in the skin, one-third of the animals (nine out of twenty-seven) evidenced degenerative changes in the liver.

3. The symptomatology and pathology resulting from a deficiency of both potassium and magnesium were similar to those of potassium deficiency, except that the rats evidenced the early hyperirritability of magnesium deficiency.

4. No abnormalities other than a subnormal rate of growth were encountered when both magnesium and potassium, in the form of the simple salts or as a part of a complex salt mixture, were added to the basal diet. In each case the rate of growth was identical.

LITERATURE CITED

BROOKFIELD, R. W. 1934 Magnesium deficiency in the rat. Brit. Med. J., vol. 1, p. 848.

CRAMER, W. 1932 Experimental production of kidney lesions by diet. Lancet, vol. 223, p. 174.

DANIELS, A. L. AND G. J. EVERSON 1936 A study of the magnesium needs of preschool children. J. Nutrition, vol. 11, p. 327.

DUNCAN, C. W., C. F. HUFFMAN AND C. S. ROBINSON 1935 Magnesium studies in calves. I. Tetany produced by a ration of milk or milk with various supplements. J. Biol. Chem., vol. 108, p. 35.

GREEN, H. H., W. M. ALLCROFT AND R. F. MONTGOMERIE 1935 Hypomagnesaemia in equine transit tetany. J. Comp. Pathol., vol. 48, p. 74.

GREENBERG, D. M. AND E. V. TUFTS 1935 a Effect of a diet low in magnesium on the rat. Proc. Soc. Exp. Biol. Med., vol. 32, p. 674.

———— 1935 b The effect of a diet low in magnesium on the rat. J. Biol. Chem., vol. 109, p. xxxviii.

GUERRANT, N. B. AND W. D. SALMON 1930 The stability of vitamin G as measured by its growth-stimulating effect. J. Biol. Chem., vol. 89, p. 199.

HIRSCHFELDER, A. D. 1933 Clinical manifestations of hypo- and hypermagnesaemia. J. Clin. Invest., vol. 12, p. 982.

———— 1934 Clinical manifestations of high and low plasma magnesium. J. Am. Med. Assoc., vol. 102, p. 1138.

HUFFMAN, C. F. AND C. W. DUNCAN 1936 Magnesium carbonate and magnesium oxide supplements to a whole milk ration for dairy calves. J. Dairy Science, vol. 19, p. 440.

KEENAN, J. A., O. L. KLINE, C. A. ELVEHJEM AND E. B. HART 1935 The stability of vitamins B(B_1), G(B_2) and B_6. J. Nutrition, vol. 9, p. 63.

KRUSE, H. D., E. R. ORENT AND E. V. MCCOLLUM 1932 a Studies on magnesium deficiency in animals. I. Symptomatology resulting from magnesium deprivation. J. Biol. Chem., vol. 96, p. 519.

———— 1932 b Chemical changes in the blood following magnesium deprivation. J. Biol. Chem., vol. 97, p. iii.

———— 1933 Studies on magnesium deficiency in animals. III. Chemical changes in the blood following magnesium deprivation. J. Biol. Chem., vol. 100, p. 603.

KRUSE, H. D., M. M. SCHMIDT AND E. V. MCCOLLUM 1934 Studies on magnesium deficiency in animals. V. Changes in mineral metabolism of animals following magnesium deprivation. J. Biol. Chem., vol. 106, p. 553.

LEROY, J. 1926 Nécessité du magnésium pour la croissance de la souris. Compt. rend. Soc. Biol., T. 94, p. 431.

LEULIER, A. AND G. VANHEMS 1934 Influence du potassium sur la croissance du rat blanc. Comp. rend. Soc. Biol., T. 115, p. 57.

MCCOLLUM, E. V. AND E. R. ORENT 1931 Effects on the rat of deprivation of magnesium. J. Biol. Chem., vol. 92, p. xxx.

MILLER, H. G. 1923 Potassium in animal nutrition. II. Potassium in its relation to the growth of young rats. J. Biol. Chem., vol. 55, p. 61.

MOORE, L. A., L. B. SHOLL AND E. T. HALLMAN 1936 Gross and microscopic pathology associated with low blood magnesium in dairy calves. J. Dairy Science, vol. 19, p. 441.

ORENT, E. R., H. D. KRUSE AND E. V. MCCOLLUM 1932 Studies on magnesium deficiency in animals. II. Species variation in symptomatology of magnesium deprivation. Am. J. Physiol., vol. 101, p. 454.

———— 1934 Studies on magnesium deficiency in animals. VI. Chemical changes in the bone, with associated blood changes, resulting from magnesium deprivation. J. Biol. Chem., vol. 106, p. 573.

PULLES, H. A. 1931 Iets over de intraveneuze infusie van geneesmiddelen, special bij grastetanie en Paresis puerperalis bovis. Tijdschr. Diergeneesk., vol. 58, p. 1334 (Nutr. Abst., vol. 1, p. 825).

ROBERTSON, E. C. AND M. E. DOYLE 1935 Intestinal stasis in low mineral diets. J. Nutrition, vol. 9, p. 553.

SALMON, W. D. AND J. G. GOODMAN 1934 Studies of the raw egg white syndrome in rats. J. Nutrition, vol. 8, p. 1.

SJOLLEMA, B. 1932 a Nutritional and metabolic disorders in cattle. Nutr. Abst., vol. 1, p. 621.

———— 1932 b Onderzoekingen over de oorzaken van grastetanie. Tijdschr. Diergeneesk., vol. 59, p. 329 (Nutr. Abst., vol. 2, p. 201).

———— 1932 c Magnesium-gebrek als oorzaak van excitatie-verschijnselen en tetanie. Tijdschr. Diergeneesk., vol. 59, p. 1052 (Nutr. Abst., vol. 2, p. 651).

SJOLLEMA, B. AND L. SEEKLES 1932 Der Magnesiumgehalt des Blutes, besonders bei Tetanie. Klin. Wochenschr., Bd. 11, S. 989 (Nutr. Abst., vol. 2, p. 291).

PLATES

PLATE 1

EXPLANATION OF FIGURES

A Rat no. 6403. Potassium deficient diet. Photomicrograph showing the type of lesion found in the tissue intervening between the 'beaded' portions. Note the distention at the tips of the villi and the rarefaction of the submucosa at this point. A few cells have accumulated at the tips of the villi, and the epithelium at this point is edematous. Note also the relative scarcity of lymphocytes and phagocytes in the lumen of the intestine as compared with B. Hematoxylin-Triosin. 15 μ. Magnification $\times$ 160.

B Rat no. 6403. Potassium deficient diet. Photomicrograph showing the type of change consistently found in the 'beaded' portions of the lower jejunum and ileum. Note the markedly edematous and vacuolated epithelium at the tips of the villi, and their marked distention with lymphocytes and phagocytes. Numbers of these cells are also to be seen within the lumen of the intestine. Hematoxylin-Triosin. 15μ. Magnification $\times$ 160.

C Rat no. 6541. Potassium deficient diet. Photomicrograph showing the marked edema and rarefaction of the whole submucosa with uniform distention of the villi. This type of change was found in the same general areas as B. Hematoxylin-Triosin. 15 μ. Magnification $\times$ 160.

D Rat no. 6541. Potassium deficient diet. Photomicrograph of a section of the wall of the left ventricle of the heart showing the type of lesion consistently found. Note the marked scarring of the myocardium. Hematoxylin-Triosin. 15 μ. Magnification $\times$ 160.

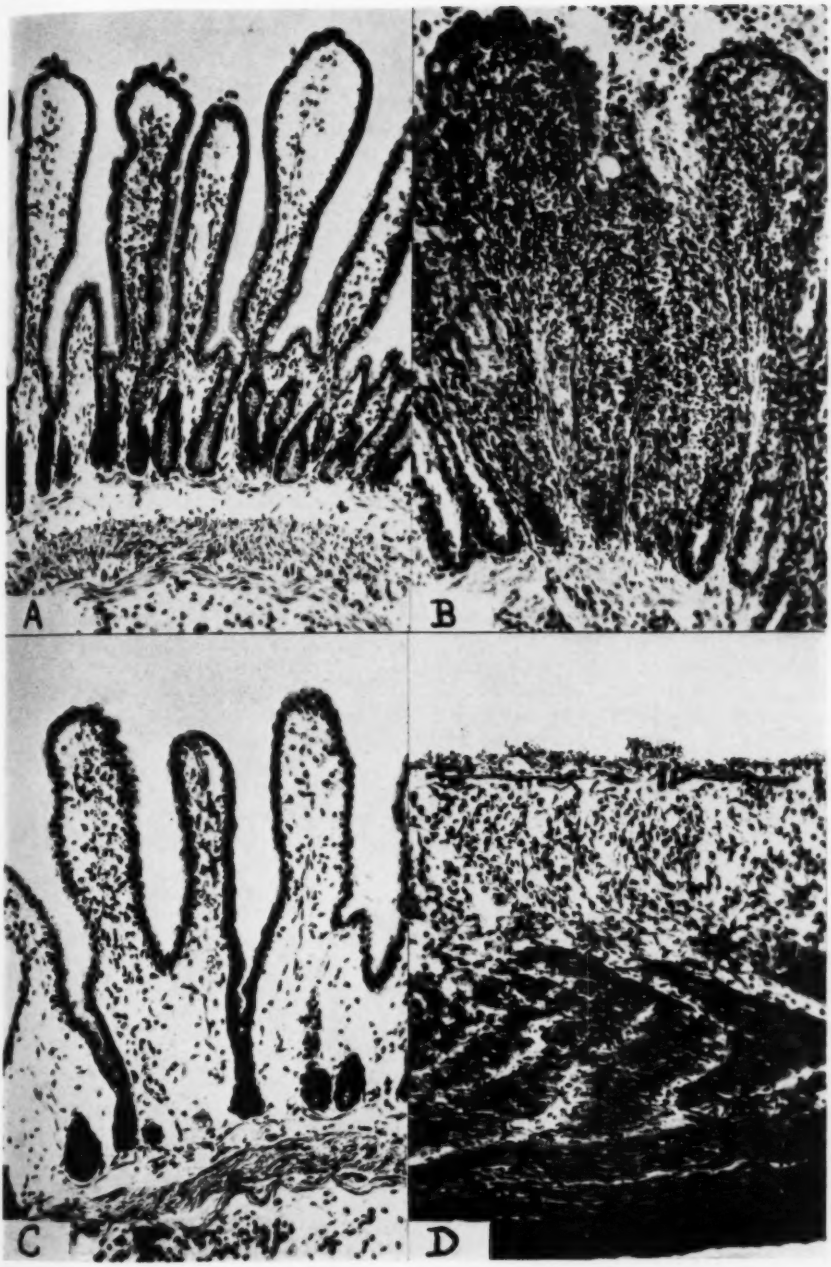

A
B
C
D

PLATE 2

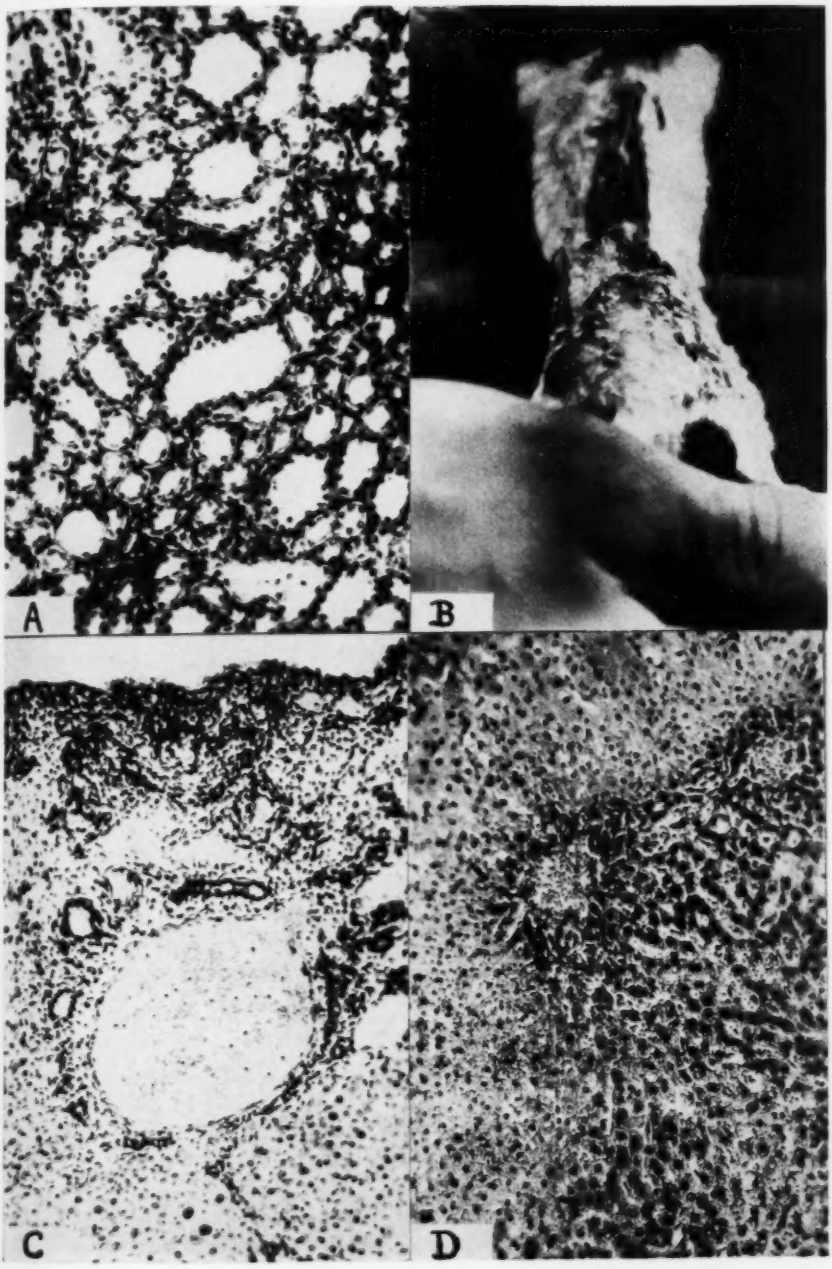
EXPLANATION OF FIGURES

A Rat no. 6551. Potassium deficient diet. Photomicrograph of a section of the kidney showing the marked dilatation of the tubules and necrosis of the tubular epithelium. Hematoxylin-Triosin. 15 μ. Magnification $\times$ 160.

B Rat no. 6731. Magnesium deficient diet. Photograph of a rat showing the marked escharotic lesions of the back and flanks.

C Rat no. 6558. Magnesium deficient diet. Photomicrograph of a section of liver showing the type of change found in those livers which had shown the cicatrizing type of lesion. Note the marked scarring and infiltration of lymphocytes and phagocytes. Hematoxylin-Triosin. 15 μ. Magnification $\times$ 160.

D Rat no. 6576. Magnesium deficient diet. Photomicrograph of a section of liver which had shown the mosaic type of lesion. Note the marked cellular destruction and the pycnosis of remaining nuclei. Hematoxylin-Triosin. 15 μ. Magnification $\times$ 160.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